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The Role of Reactive Oxygen Species in the Induction of Interleukin-8 (IL-8) by Rhinovirus

Matthew Biagioli
Medical University of South Carolina

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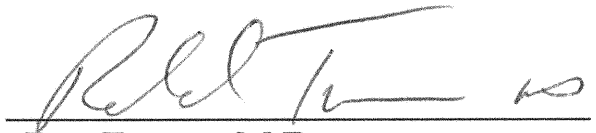
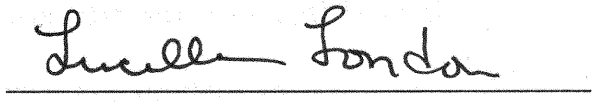
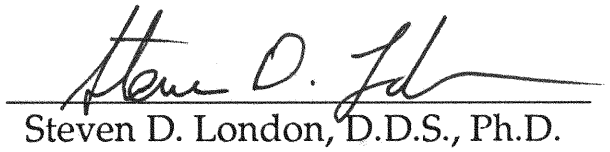


Ron Turner, M.D.

Salvatore Arrigo, Ph.D.



Lucille London, Ph.D.



Steven D. London, D.D.S., Ph.D.

Steven Rosenzweig, Ph.D.

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LISTS OF ABBREVIATIONS

BEBM	Bronchial Epithelial Basal Medium
BEGM	Bronchial Epithelial Growth Medium
C5a	Complement Component 5a
Con A	Concavalin A
DCFDA	Dichlorofluorescein Diacetate
DMPO	5'5'-Dimethyl-1-Pyrroline N-Oxide
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme Linked Immunosorbant Assay
EMEM	Eagles Minimum Essential Medium
EMSA	Electrophoretic Mobility Shift Assay
FMLP	Formyl-methionyl-leucyl-phenylalanine
GSH	Glutathione
H_2O_2	Hydrogen peroxide
hRV	human Rhinovirus

IL-1	Interleukin-1
IL-8	Interleukin-8
INF	Interferon
LPS	Lipopolysaccharide
LTB_4	Leukotriene B4
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NAC	N-Acetyl Cysteine
NAP-1	Neutrophil-activating protein-1
O_2-	Superoxide anion
$OH^{\bullet}$	Hydroxyl radical
PAF	Platelet Activating Factor
PBN	α-Phenyl-tert-butyl Nitrone
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PMNs	Polymorphonuclear Leukocytes
ROS	Reactive Oxygen Species
$TCID_{50}$	Tissue Culture Infectious Dose 50
TNF-α	Tumor Necrosis Factor-α
WBC	White Blood Cells

SUMMARY

There is a direct correlation between the severity of common cold symptoms and the concentration of IL-8 in nasal secretions from volunteers with experimental human rhinovirus (hRV) colds. Although these and other data are consistent with the hypothesis that IL-8 is a mediator of common cold symptoms, the mechanism by which IL-8 is elaborated in response to hRV challenge is not understood. This study examined the role of oxidative stress and NF-κB activation in hRV induced IL-8 elaboration. All studies were done in either a transformed respiratory epithelial cell line (Beas-2b) or human lung fibroblasts (MRC-5). Induction of oxidative stress by hRV was demonstrated by fluorescent staining of carbonyl groups, conversion of dichlorofluorescein diacetate (DCFDA) to fluorescein , and H_2O_2 production. H_2O_2 concentrations were 4.74 ± 2.04 μM and 0.40 ± 0.49 μM in supernatants from hRV challenged and control Beas-2b cells, respectively ($p<0.01$, Wilcoxon sign rank). Treatment of both MRC-5 and Beas-2b cells with exogenous H_2O_2 resulted in a dose dependent elaboration of IL-8. Antioxidants attenuated IL-8 secretion by viral treated cells. Treatment of Beas-2b and MRC-5 cells with N-acetyl cysteine (NAC) inhibited hRV induced IL-8 elaboration. IL-8 concentrations in supernatants from Beas-2b cells were $35.8 \pm$

2.1 pg/ml and 10.0 ± 1.0 after virus challenge in untreated and treated cells (30 mM NAC) respectively. In MRC-5 cells viral stimulation of IL-8 was reduced from 403.8 ± 85.7 pg/ml to 239 ± 67.7 pg ml IL-8 ($p<0.02$, Wilcoxon sign rank) by treatment with 20 mM NAC. The antioxidants, dimethyl sulfoxide (DMSO) and ferulic acid also inhibited viral-induced IL-8 in both cell lines; two other antioxidants, 5'5'-Dimethyl-1-Pyrroline N-Oxide (DMPO) and α-Phenyl-tert-butyl Nitrone (PBN) had little effect on IL-8 elaboration. The production of IL-8 in response to oxidative stress appeared to be mediated by NF-κB . NF-κB was activated in virus challenged Beas-2b cells but not in unchallenged cells and this activation was inhibited by treatment with 20 mM NAC. Activation of NF-κB was also seen in Beas-2b cells following treatment with 1 mM H_2O_2. These data suggest that hRV stimulation of IL-8 is mediated through the production of reactive oxygen species (ROS) and their subsequent activation of NF-κB. The antioxidants NAC, DMSO, and ferulate inhibit hRV induced IL-8 elaboration although the precise mechanism of this inhibition has not been determined.

CHAPTER 1

LITERATURE REVIEW

1. Common Cold Pathogenesis

The common cold is a leading cause of absence from school and work resulting in an estimated 26 million days of school absence and 23 million days of work absence in the U.S. alone (25). The complications of the common cold, otitis media, exacerbation of chronic bronchitis, sinusitis and asthma are associated with significant morbidity (50). Human rhinoviruses (hRV) are the causative agent in 33-50% of all common colds (44,53). In spite of the importance of rhinovirus as a human pathogen, the pathophysiology of rhinovirus infection is poorly understood.

Early studies of hRV infection found little evidence of histopathological damage and suggested that the cellular immune response may be important in symptom production (reviewed in Turner, 71 & 74). Human volunteers who are experimentally infected with hRV do not consistently develop common cold symptoms (Figure 1) (44). Approximately 25-33% of subjects who are infected with hRV in this model do not meet a standard definition for a common cold (44,73,79). Comparison of the infected symptomatic with the

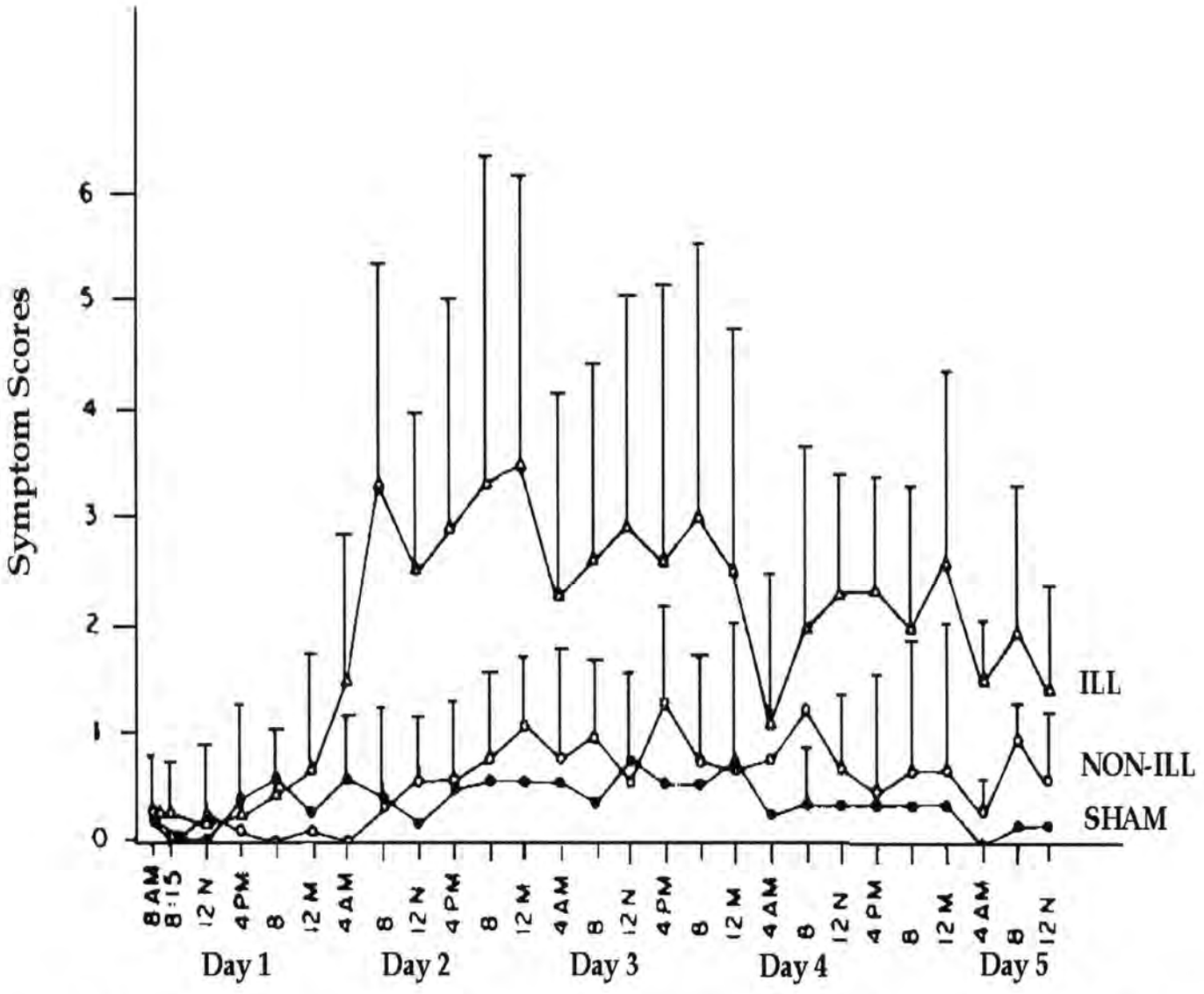

Figure 1. Symptom scores (mean ± SD) in rhinovirus-infected and sham challenged subjects. Infected subjects were separated into ill and non-ill groups by a modification of the criteria of Jackson et al. (1958) (28). P=.002 for comparison of the ill and control groups; other comparisons were not significant. (Δ), Infected, ill group (n=11); (o), infected, non-ill group (n=8); (•), control group (n=5). (Modified from *J. Infect. Dis.* **157**(1). 136.)(44)

infected "asymptomatic" subjects in these studies has provided some useful insights into the pathogenesis of common cold symptoms during hRV infection (44,53,73,79).

Among infected individuals, those who develop illness have an increase in peripheral white blood cell (WBC) count (53). This rise in WBCs is seen in the first 2-3 days following intranasal inoculation with hRV and is the result of an increase in the number of circulating PMNs (33,53). Infected non-ill individuals show no detectable change in WBC count (53).

In association with the peripheral cellular response there is also a cellular response in the nasal mucosa and nasal secretions of individuals with hRV colds. Neutrophils infiltrate nasal mucosa and appear in nasal secretions early in the course of the cold (47,79). In a study of natural colds, PMNs were significantly increased in nasal mucosal biopsies on the second day of illness when compared to specimens taken from the same individuals two weeks later or from asymptomatic individuals at either time point (79). These results were confirmed by a later study with experimental hRV in which a significant rise in PMNs was seen in the nasal epithelium on days 3 and 4 after virus challenge (80). This PMN infiltration of the nasal epithelium is paralleled by a increase in PMN concentration in nasal secretions (33,44). In a study performed by Naclerio et al. (1988) it was observed that healthy individuals who were infected with experimental hRV and developed symptoms had increased neutrophil counts in nasal lavages beginning on day 3 of the study (44). This early PMN increase was not seen in either infected asymptomatic or sham inoculated volunteers (Figure 2) (44). These increases of PMNs in nasal secretions, as with PMN increases in peripheral blood, are associated with illness rather than infection (44,80) . The association of PMN infiltration with symptoms suggests that the host response to the virus may play a role in common cold symptom production.

Initial studies of the mechanism of PMN recruitment to the nasal epithelium found that infection of embryonic lung fibroblast cells with hRV stimulated elaboration of a chemoattractant for PMNs (70). Subsequent studies revealed that upon hRV

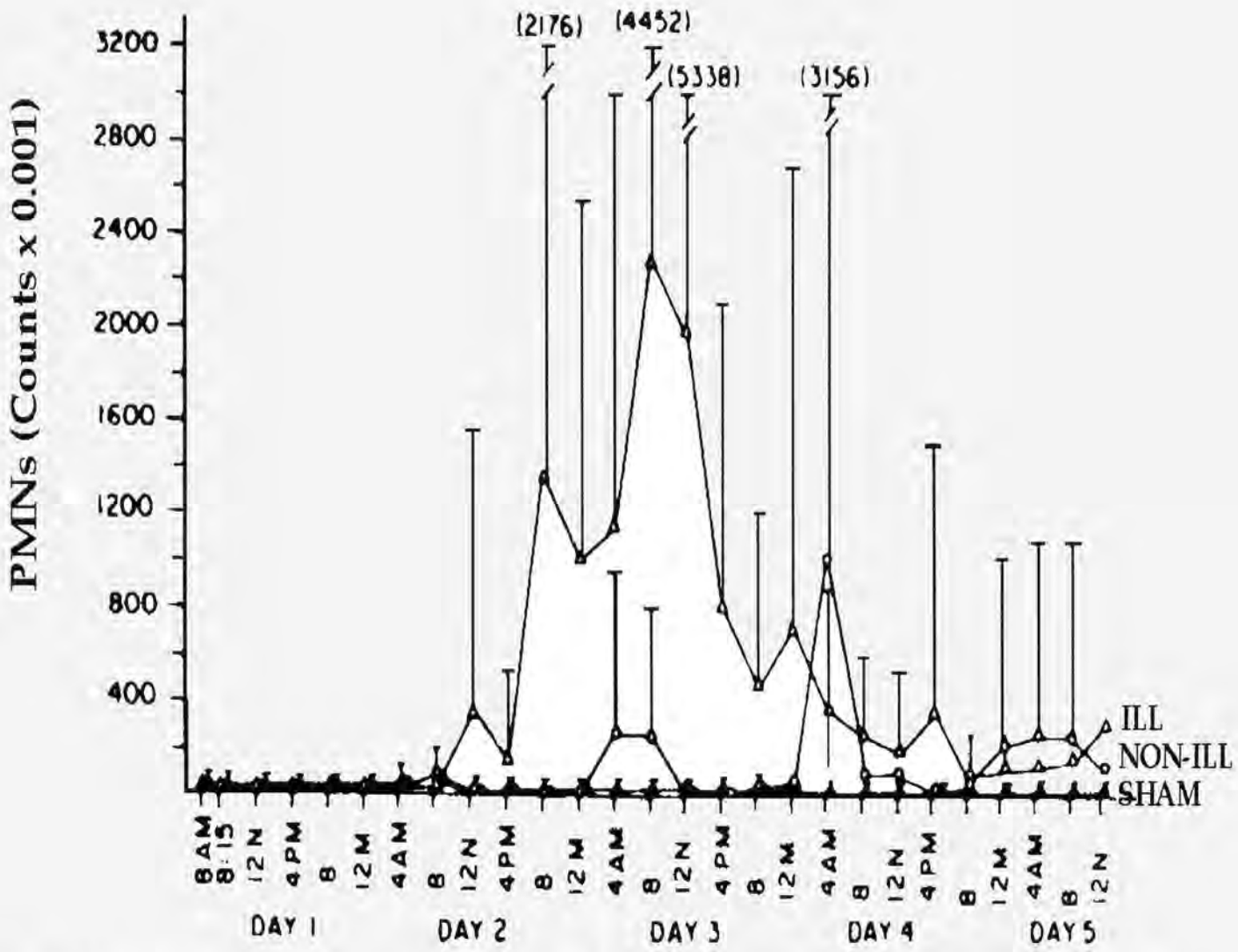

Figure 2. Neutrophil counts/mL (mean ± SD) in nasal lavages of rhinovirus-infected and sham challenged (n=7) subjects. Infected subjects were separated into ill (n=12) and non-ill (n=10) groups by a modification of the criteria of Jackson et al. (1958) (28). P<.01 for comparison of the ill and control groups; other comparisons were not significant. Symbols are the same as for figure 1. (Modified from *J. Infect. Dis.* **157**(1). 138.)(44)

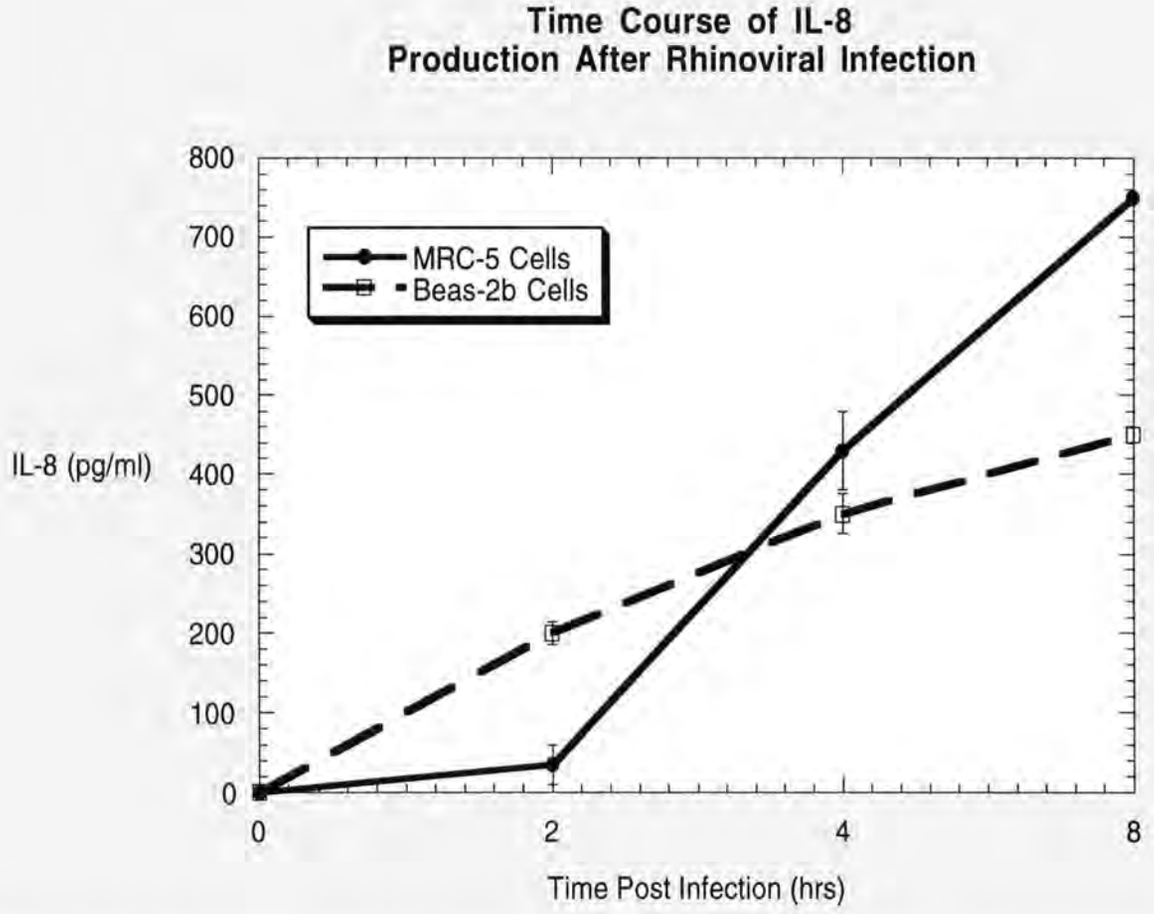

Figure 3. IL-8 concentration(mean ± SE) in supernatants collected from a human lung fibroblast cell line, MRC-5, and a human bronchial epithelial cell line, Beas-2b, after infection with hRV. Supernatants were collected at times 0, 2, 4, and 8 after viral challenge. (Modified from J. Clin. Invest. **96**: 549-57 and unpublished data) (66)

challenge, MRC-5 cells, a human lung fibroblast cell line, and Beas-2b cells, a transformed bronchial epithelial cell line, elaborate interleukin-8 (IL-8). (3,9,11,48,73) (Figure 3). IL-8 has also been found in nasal secretions from volunteers infected with experimental hRV and from children with naturally acquired respiratory infections (47,72,73). A study performed by Turner et al. (1998) in which healthy individuals were infected with hRV demonstrated that infected symptomatic individuals had significantly increased IL-8 concentrations in nasal secretions on days 2-4 following hRV challenge compared to either infected asmyptomatic or sham inoculated individuals (73) (see Table).

Study Day	Infected total symptom score > 6 (n=37)	Infected total symptom score < 6 (n=17)	Sham inoculated (n=20)
0	67(16:161)	63(35:100)	51(16:238)
1	66(38:128)	58(32:141)	72.5(16:136)
2	269(130:530)	109(50:198)	77(44:222)
3	349(146:701)	192(40:220)	104(57:185)
4	330(137:705)	120(36:194)	71(39:132)

Table 1. Nasal Wash IL-8 concentrations in Rhinovirus infected and sham challenged subjects. The median for the sample is indicated by the number preceding the parentheses. Inside the parentheses represent the 25th and 75th percentiles, respectively. Nasal lavage IL-8 concentrations were significantly greater in those subjects with symptomatic infections than in those with asymptomatic infections on study days 2 ($P = .008$), 3 ($P = .004$), and 4 ($P = .003$) by Mann-Whitney U test. The IL-8 concentrations in subjects with asymptomatic infections were not significantly different from those in the sham challenged subjects (Modified from Turner, 1998)(73).

In addition, this study found a direct correlation between the severity of nasal obstruction, rhinorrhea and total symptoms and the concentration of IL-8 in nasal secretions from volunteers infected with experimental hRV (73). The association between IL-8 elaboration and symptoms was further suggested by a study of experimental intranasal challenge of healthy volunteers with IL-8. Douglass et al. (1994), demonstrated that there was an increase of PMNs in nasal smears, and a transient production of cold symptoms following intranasal challenge with IL-8 (18). The increase in IL-8 among infected symptomatic individuals but not infected asymptomatic individuals, the correlation between symptom severity and IL-8 concentration, and the production of cold symptoms upon challenge with IL-8 all support the hypothesis that IL-8 is a mediator of hRV colds. Our current hypothesis is that upon hRV infection of the nasal epithelium, there is an elaboration of IL-8 by these epithelial cells. This IL-8 then acts as a chemoattractant for PMNs. The migration of PMNs is associated with transudation of serum that results in elaboration or activation of other inflammatory mediators (Figure 4) leading to the precipitation of common cold symptoms.

2. Interleukin-8 (IL-8)

IL-8 was originally characterized as neutrophil-activating protein-1 (NAP-1) with biological properties similar to known chemotactic substances such as the plasma-derived anaphylatoxin C5a, the cell derived chemotaxins leukotriene B_4 (LTB_4), platelet activating factor (PAF), and bacterial- or synthetic formylmethionyl peptides (e.g. FMLP). IL-8 is one of several proinflammatory

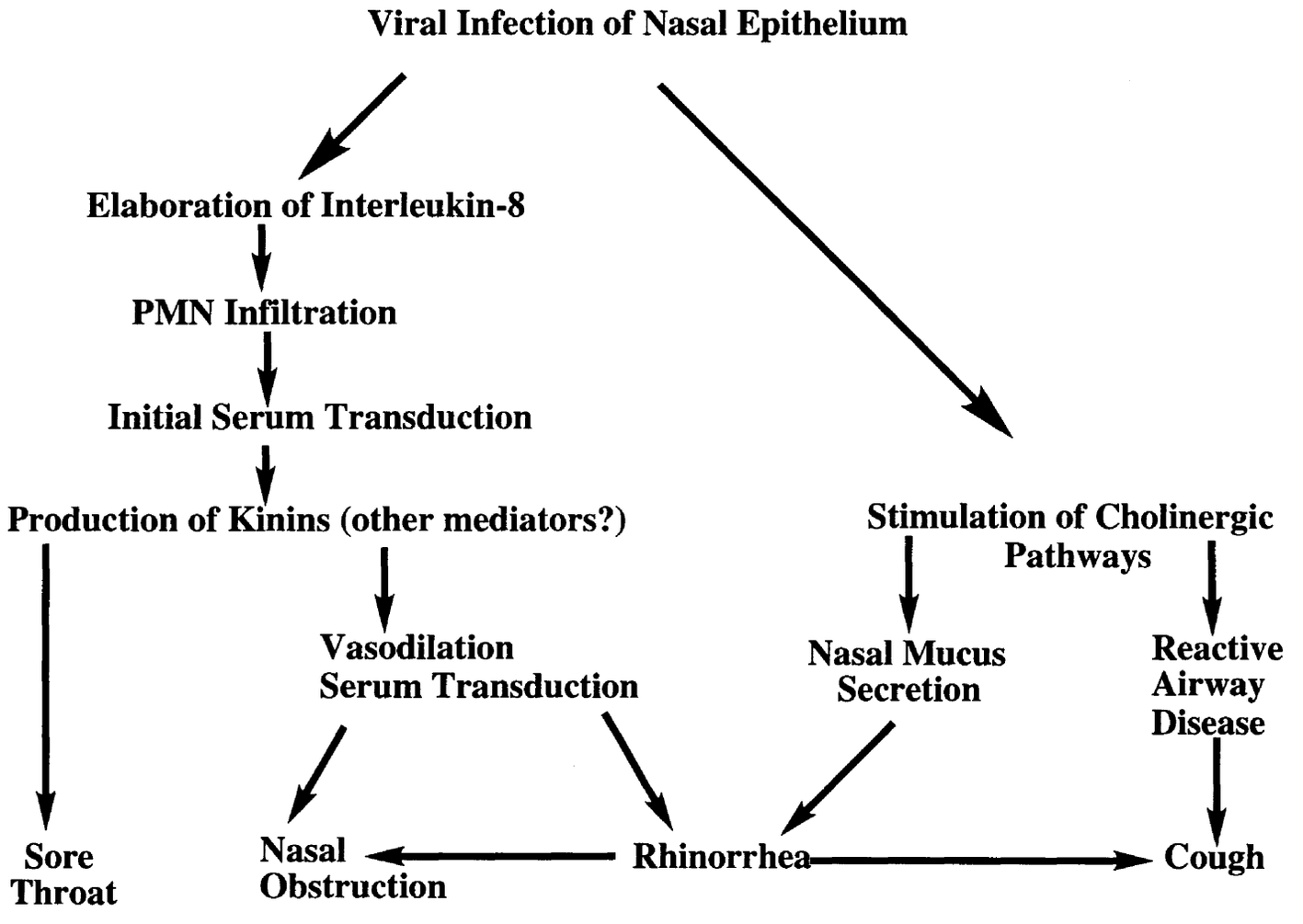

Figure 4. Hypothetical pathogenesis of rhinovirus infection of nasal epithelium ultimately resulting in common cold symptoms.

cytokines that are characterized by the conservation of four cysteine residues that are important for tertiary structure. IL-8 can be produced by a variety of cells, including leukocytes, fibroblasts, endothelial cells, chondrocytes, keratinocytes and, of particular relevance to this study, epithelial cells in response to a number of stimuli (76). In epithelial cells, IL-1, virus (hRV and others), TNF-α, elastase, and PMA have been shown to induce IL-8 (46,65).

The most potent action of IL-8 is as a chemoattractant for PMNs. High affinity receptors have been identified on the surface of human neutrophils that bind IL-8 (58). Low numbers of these receptors have been found on other leukocytes, including T lymphocyte and monocyte cell lines (39). *In vitro* , IL-8 is a potent chemoattractant for neutrophils and also has chemotactic activity for T lymphocytes and basophils. IL-8 also has other effects on PMN function. It induces neutrophil degranulation measurable by the release of granule constituents (51,61) and produces a rapid increase of cytoplasmic-free Ca^{2+} (67). IL-8 also stimulates phagocytosis of opsonized particles (15), enhances the adhesion of neutrophils to the endothelium (14), and induces the trans-endothelial migration of neutrophils (22), but induces only a weak respiratory burst through the formation of superoxide and hydrogen peroxide (61). Intradermal injection of IL-8 causes infiltration by neutrophils as well as lymphocytes (31), and its intra-articular administration induces destruction of the synovium in a neutrophil-dependent fashion (19). The administration of neutralizing antibody to IL-8 inhibits neutrophil infiltration and tissue damage in several models of inflammation (27).

3. Genetic Regulation of IL-8

Mukaida et al. (1996) cloned and sequenced the IL-8 gene including the 1.5 kb 5′-flanking region. The IL-8 gene consists of four exons and three introns with a single TATA- and CAT- like structure up stream of the coding region (40). An S1 nuclease protection assay revealed that there is a single transcription start site (40). The 5′ flanking region of the IL-8 gene shows no overall sequence similarity to other cytokines or acute phase reactant proteins whose expression is also induced by IL-1. However, the 5′ flanking region contains several potential binding sites for known transcription factors such as AP-1, NF-IL6, and NF-κB (Figure 5). Deleted or mutated 5′ flanking regions were cloned into a chloramphenicol acetyltransferase (CAT) expression vector and then transfected into a human glioblastoma cell line, T98G (43). This analysis revealed that the region from -94 to -71 bp, consisting of NF-IL6 and NF-κB binding sites, is minimally required for responsiveness to IL-1. This same combination of cis acting elements were required for IL-8 gene activation in the human fibrosarcoma cell line, 8387, stimulated with IL-1 or TNF-α (41).

IL-8 gene activation may also be mediated by the AP-1 binding site (-126 to -120 bp) in conjunction with the NF-κB (42). The requirement for this small region was shown in Jurkat cells and was confirmed in human lung epithelial cell lines and a human gastric cancer cell line, MNK45 cells (49,81). Using electrophoretic mobility shift assay (EMSA) for the IL-8 gene in MNK45 cells, activation of both AP-1 and NF-kB were found, however, no activation was detected at the NF-IL6 site (45). Moreover, when T98G cells were transfected with a IL-8 gene-CAT expression vector containing a mutated NF-IL6 and intact AP-1 and NF-kB sites, CAT activity was induced by the stimulation with interleukin-1

(43). Recently, in a study performed by Zhu et al. (1997), using a IL-8 promoter-driven luciferase assay, it was demonstrated that hRV is a potent stimulator of IL-8 promoter-luciferase activity and that this stimulation is abrogated by the mutation of the NF-κB site in the promoter construct (83). Taken together, these observations suggest that although NF-κB is the most crucial factor for IL-8 gene transcription, cooperation with another type of transcription factor is necessary (Figure 6). NF-IL6 is the preferred operator, while AP-1 can substitute in either the absence of NF-IL6 or if the NF-IL6 site is mutated. However, in all cell types examined, NF-κB is absolutely required for IL-8 gene transcription (38,42,43,45,49,83).

4. NF-κB Relationship with Reactive Oxygen Species (ROS)

NF-κB was first identified as a DNA binding protein with activity specific for the κB motif in the immunoglobulin kappa light chain enhancer in B lymphocytes (62). Decameric κB-like recognition sites have since been found in many genes involved in immune function and acute phase responses (21). The cloning of the genes encoding the two subunits of NF-κB, p50 (NFKB-1) and p65 (relA), revealed a family of NF-κB/rel proteins that participate in various transcriptionally controlled processes, such as cytokine responsiveness, lymphoid differentiation and embryonic axis determination in insects(32).

NF-κB/rel proteins are subject to multiple regulatory influences. A major component of this regulation is the control of their intracellular localization, with inactive protein maintained in the cytoplasm and transcriptionally active protein transported to the nucleus. Cytoplasmic retention of the NF-κB/rel

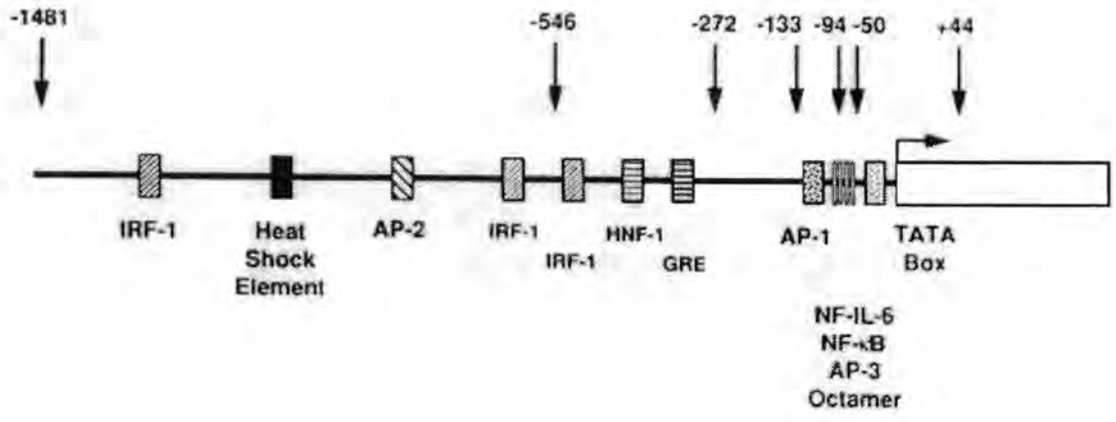

Fig.5. Schematic representation of 5'-flanking region of IL-8 gene, demonstrating locations of several known binding sites for various transcription factors (IRF = INF Regulatory Factor, Ap = Activator protein, HNF = Hepatocyte Nuclear Factor, GRE = Glucocorticoid Response Element). (Mastronarde et al. 1996. *J. Infect. Dis.* **174**:262-267)(38)

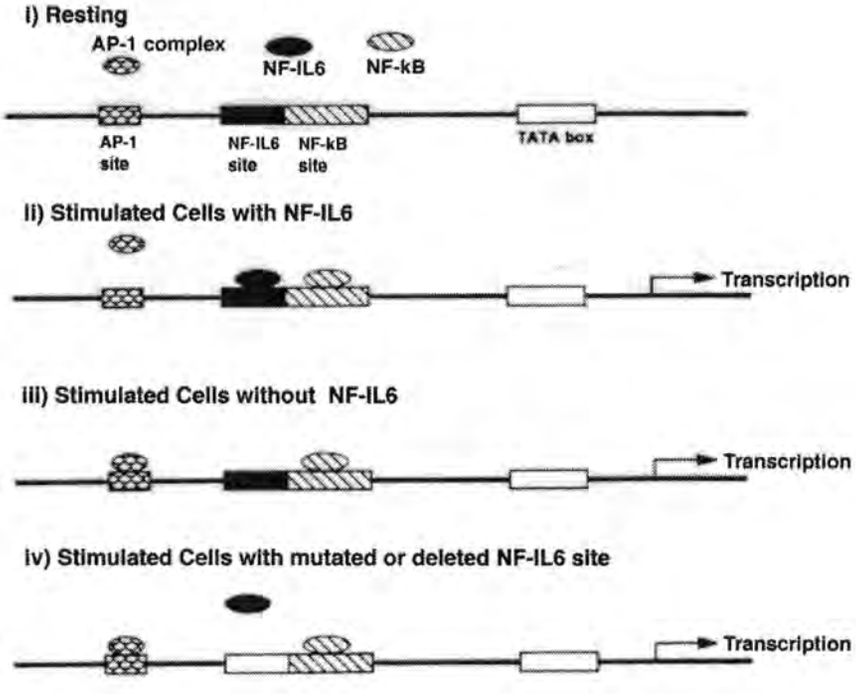

Fig.6. Proposed mechanism of regulation of IL-8 transcription by AP-1, NF-IL6, and NF-κB. (Mukaida et al. 1994, *J Leuk. Biol.* **56**:554-558)(42)

proteins is achieved through interaction with a group of inhibitor proteins, the I-κBs (42). NF-κB is regulated through phosphorylation and or proteolytic cleavage of I-κB.

In recent years, an association between cellular ROS generation and NF-κB activation has been found. Molecular oxygen is essential for respiration by aerobic organisms. Through incomplete electron transfer on O_2 or by the activity of various enzymes, partially reduced forms are produced (see below). These ROS are extremely hazardous and include the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the most powerful oxidant, the hydroxyl radical ($OH^\bullet$).

$$4e^- + 2O_2 \text{ --> } 2O_2^\bullet + 2H^+ \text{---> } H_2O_2 \text{ ---> } H_2O + O_2 \text{ or } OH^-$$

Since ROS are continuously produced in living cells, a certain 'normoxic' concentration of ROS is physiologic. Above normal concentrations of ROS within a cell induce a state called 'oxidative stress'. Cells can also selectively generate specific ROS. For example, both the oxidative burst in macrophages and neutrophils during inflammation and TNF-α mediated cell killing rely on *de novo* synthesis of ROS (63). Furthermore, ROS are also involved in intracellular signaling processes, as they modify the activity of proteins on the post-translational level and regulate gene expression (2).

Recent studies have demonstrated that the activities of NF-κB and AP-1 can be regulated by changes in the redox state of the cell. Schreck et al. (1991) proposed that NF-κB activation is mediated by the production of ROS, which act as a type of second messenger (60). This hypothesis was originally supported by the observations that NF-κB inducers TNF-α, IL-1, PMA, UV light and γ-rays are known to induce oxidative stress in cells (2,16). Schreck and

coworkers addressed the question of how such diverse agents could trigger the same reaction, that is, the release of the I-κB inhibitor from the NF-κB protein complex. Earlier work had shown that in HIV-infected lymphoma T cell lines, PMA or TNF-α could activate the enhancer/promoter LTR of the virus. Sappey et al. (1995) also observed that virus expression was stimulated after a short stress with H_2O_2 (59). This activation could be inhibited by the addition of certain antioxidants. It was also found that such inhibition was correlated with a lack of NF-κB activation. Schreck et al. (1991) performed further experiments on Jurkat T cells (60). These cells can also be activated by several factors such as PMA, TNF-α and IL-1 which induce NF-κB binding to DNA (60). The seminal observation from these studies was that direct application of low concentrations of H_2O_2 to the Jurkat T cells was able to similarly activate the NF-κB protein. The mechanism by which ROS participate in NF-κB activation is yet to be determined (23).

In summary, it has been found that PMA, IL-1, and TNF-α all cause a state of oxidative stress in cells. In addition these compounds all activate NF-kB, and their activation of NF-kB can be inhibited by antioxidants. Furthermore, NF-κB is activated in Jurkat T cells by treatment with the ROS H_2O_2,. These observations suggest that NF-κB activation can be mediated by oxidative stress. Since NF-kB activation is essential for transcription of IL-8, it is plausible that oxidative stress may be a mediator of hRV induced IL-8 elaboration.

5. Focus of this Study

The specific aim of this study was to measure the effects of hRV on ROS, and their subsequent effect on NF-κB activation. This aim was addressed by experiments directed at five separate questions. 1) Does oxidative stress up

regulate IL-8 in cells of human respiratory origin? This question was addressed by exposing cells to ROS and measuring IL-8 elaboration.

2) Does rhinoviral challenge of these cells causes oxidative stress? Oxidative stress following virus challenge was accessed by a fluorescent carbonyl detection system and then through the treatment of infected cells with dichlorofluorescein diacetate which gives a fluorescent signal in the presence of oxidative stress, as well as measuring H_2O_2 production by cells challenged with hRV.

3) Does challenge of a human respiratory cell line with either hRV or ROS result in NF-κB activation? This was investigated by EMSA using a NF-κB probe.

4) Do antioxidants inhibit hRV induced NF-kB activation and IL-8 elaboration? This was studied through attempting to inhibit viral elaboration of IL-8 or NF-κB activation by pretreatment of virally infected cells with antioxidants.

Despite the prevalence of hRV infections, the pathophysiology is still poorly understood. This study attempts to distinguish those events initiated by hRV that lead to IL-8 secretion. These events may prove clinically significant as potential targets for inhibition of the inflammatory process and as a treatment of common cold symptoms.

CHAPTER II

EXPERIMENTAL PROCEDURES

1. Cell Culture

All experiments were performed using human respiratory cell lines. Diploid human embryonic lung fibroblast cells (MRC-5, Biowhittaker, Walkersville, MD) were grown in Eagles Minimal Essential Medium (EMEM) supplemented with 10% fetal calf serum , penicillin (5 μg/ml), and streptomycin (5 units/ml). MRC-5 cells were used for experiments when 100% confluent at passage 21-26. Human bronchial epithelial cells (Beas-2b, ATCC, Rockville, MD) were produced from cells isolated from human bronchial epithelium obtained from autopsy of non-cancerous individuals. The cells were infected with an adenovirus 12-SV40 virus hybrid (Ad12SV40) and cloned (75). Beas 2B cells were grown in bronchial epithelial growth media (BEGM, Clonetics, Minneapolis, MN) supplemented with human recombinant epithelial growth factor (0.5 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), epinephrine (0.5 μg/ml), transferrin (10 μg/ml), gentamicin (50 μg/ml), and amphotericin B (50 ng/ml). The cells were passed to 6, 12, 24, or 96 well tissue culture plates and used for experiments when they reached 85-95% confluence except where indicated. All experiments with Beas-2b cells were done with cells at passage 35-55.

2. Viral preparation and purification

Rhinovirus type 39 (hRV-39), a rhinovirus serotype that is known to cause IL-8 elaboration in vivo and is one of the serotypes dependent on intracellular adhesion molecule-1 (ICAM-1) for cellular attachment, was used for all experiments (69). hRV-39 was grown in HeLa-I cells, a HeLa clone that has been found to have increased surface expression of ICAM-1 (provided by F.G. Hayden, University of Virginia HSC, Charlottesville, VA). HeLa-I cells infected with hRV39 were mechanically collected, lysed by a freeze-thaw and clarified by centrifugation at 2000 g (Beckman GPR centrifuge, Beckman Instruments, Inc., Palo Alto, CA). The supernatants were then centrifuged at 126,086 g at 4° C for 45 min using a Ti45 rotor (Beckman L8-70M centrifuge, Beckman Instruments, Inc., Palo Alto, CA) . For experiments with unpurified virus, the resulting viral pellet was resuspended in EMEM and stored at -80° C in 500 µl aliquots. Purified virus was produced by a modification of a published method (82). Briefly, after ultracentrifugation, the resulting viral pellet was resuspended in 200 µl of PBS and overlaid onto a two layer sucrose cushion containing 60% sucrose in PBS on the bottom layer and 30% sucrose in PBS on the top layer. Following centrifugation at 112,700 g (SW28 rotor) for 135 min at 4° C, the interface containing the virus was collected and resuspended in 50 ml of EMEM. The virus suspension was again centrifuged at 126,086 g for 45 min at 4° C and the resulting pellet was resuspended in EMEM containing 1% BSA and aliquots were stored at -80 ° C.

3. Detection of oxidative stress

Oxidative stress in virus -challenged cells was assessed with two different assays.

Carbonyl Assay: Oxidative stress in cells results in increased number of carbonyl groups. Detection of these carbonyl groups by a previously described method (32) was used as one measure of oxidative stress in virus-challenged cells. Beas-2b cells were grown on 18 mm circle coverslips in 12-well tissue culture plates (Becton Dickinson & Co., Lincoln Park, NJ). When the cells were 50% confluent, they were washed 2 times with BEBM and then challenged with hRV39 at a multiplicity of infection (MOI) of 100 $TCID_{50}$/cell (tissue culture infectious dose 50% per cell). Control cells were treated with BEBM alone. Virus-challenged and control cells were incubated in the dark for two hours at 33° C. Cells incubated with 15% H_2O_2 in the dark for 15 min at 37° C were used as a positive control. Following the incubation, the cells were washed 3 times with cold PBS (pH 7.2), fixed with cold ethanol solution for 15 min at 4° C and then washed 3 times with PBS followed by a 30 min incubation in PBS at 4° C. The cells were then incubated in 5 mM biotin-amidocapryol hydrazide (Sigma Chemicals, Inc., St. Louis, MO) in 200 mM sodium acetate containing 0.5% phenol, 5 mM EDTA, and 50% PEG 200 for 1 hour at 37° C. The wells were rinsed 3 times with PBS followed by a 5 min incubation in PBS containing 1% BSA at 4° C. The PBS was removed and avidin-fluorescein, prepared as a stock solution of 0.5 mg/ml in 50% picric acid saturated water, pH 7.2, and then diluted 1:200 in PBS, was added to each well. After incubation for 15 min at 4° C, the coverslips were removed, mounted with Aqua-Mount (Lerner

Labs, CT) and examined with a confocal microscope (MRC-1000, Bio-Rad Laboratories, Hercules, CA) at 250X magnification. Images were captured as 8-10 0.64 nm slices and projected via NIH Image software (NIH, Bethesda, MD,USA).

Conversion of 2',7'-dichlorofluorescein (DCFDA) diacetate to fluorescein: The conversion of DCFDA, which is not fluorescent, to fluorescein is catalyzed by oxidative stress in the cell. For this assay, Beas-2b cells were grown on coverslips in 12-well tissue culture plates as described above. When the cells were 50% confluent, they were washed 2 times with BEBM then incubated for 15 min at room temperature in 500 µl of a 1:100 dilution of 10 µM DCFDA (Molecular Probe, Eugene, OR) in PBS (pH 7.2). After this incubation, the cells were washed 2 times with PBS (pH 7.2) then treated with cell culture medium alone or with medium containing either 5% H_2O_2, or hRV39 (MOI=100 $TCID_{50}$/cell). After incubation for 1 hr at 37° C in the dark, the cells were washed twice with cold PBS, mounted and examined for fluorescence as described above.

4. Measurement of Hydrogen Peroxide in Supernatants

The cellular production of H_2O_2 in response to virus challenge was assessed by measuring the concentration of H_2O_2 that diffused into supernatant media. Beas-2b cells were grown in 24-well tissue culture plates until they were 85-95% confluent. The cells were washed 3 times with media and then incubated in media alone for 30 min at 33° C. The cells were then challenged with hRV39 (MOI=100 $TCID_{50}$/cell) or with media alone in a volume of 200 µl and incubated at 33° C for 4 hrs. 200 µl of aminotriazole solution (4 mg/ml in media) was then added to each

well and briefly mixed by pipeting. Hydrogen peroxide was measured in the supernatants by a modification of a previously published method(52). An indicator solution (2.8 ml PBS, pH 7.2, 2 ml horseradish peroxidase at a concentration of 0.5 mg/ml in PBS, pH 7.2, and 200 ul of 28 mM phenol red in PBS, pH 7.2) was prepared and 100 µl were added to each well of a 96 well plate. An equal volume of H_2O_2 standard or sample supernatant was added to each well, incubated for ten minutes at room temperature and then the reaction was stopped by the addition of 10 µl of 1N NaOH. All assays were done in duplicate on an automated spectrophotometric plate reader (Anthos HTII, Anthos Labtec Instruments Co., Salzburg, Germany) at 610 nm. Sample H_2O_2 concentrations were determined from optical density (OD) values using a standard curve based on a linear regression. Standard values were between 0.1 and 30 µM H_2O_2.

5. Measurement of IL-8 Protein

Beas-2b and MRC-5 cells were grown in 24-well tissue culture plates. The Beas-2b cells were used when they were 85-95% confluent and the MRC-5 cells were used within two days after reaching complete confluence. The virus challenge was 100 $TCID_{50}$/cell for experiments in Beas-2b cells and 10 $TCID_{50}$/cell for experiments in MRC-5 cells. The cells were washed 3 times with media and then incubated for 60 min at 37° C with either medium alone or with medium containing the following concentrations of antioxidants: 5, 10, 20, and 30 mM of NAC; 0.5%, 1%, 2%, and 3% DMSO; 5, 50, and 500 µM DMPO; 2, 20, and 200 µg/ml PBN; or 0.0025, 0.005, 0.25, 0.5, 2.5, and 5 mM ferulate. The cells were then challenged with virus in a final volume of 1 ml/well and incubated at 33° C for 1hr to allow for the absorption of

the virus. Cells were then washed 3 times with media and brought to a final volume of 500 µl/well and incubated for 6 hrs at 33° C. The 6 hr time point was chosen because it allows for measurable IL-8 elaboration yet precedes viral replication. Supernatants were then collected and stored at -80° C until analyzed for IL-8 protein. For experiments with hydrogen peroxide, supernatants were collected after the cells were challenged with H_2O_2 at 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 mM concentrations for 6 hrs at 33° C. The IL-8 concentrations in the cell culture supernatants were determined by ELISA using a commercially available assay (Quantikine, Minneapolis, MN). All assays were done in duplicate on an automated spectrophotometric plate reader (Anthos HTII, Anthos Labtec Instruments Co., Salzburg, Germany). Sample concentrations were determined from OD values using a standard curve based on a linear regression. The measurement range of the standard curve was between 8 - 2000 pg/ml. None of the antioxidants used had a demonstrable effect on the detection of IL-8 .

6. Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared by a modification of the method described by Dignam et al. (17). Beas-2b cells were grown in 6 well plates until they were 90-95% confluent (10^7 cells/well). The cells were incubated for 30 min at 37° C with either media alone, media containing 20 mM NAC (pH 7.5) or media containing 3% v/v DMSO. After incubation, the media was removed and the cells were exposed to media alone, media containing hRV39 (MOI=100 $TCID_{50}$/cell) , or media containing H_2O_2 (1 mM) for 2 hrs at 33° C. The cells were then washed twice with cold PBS, harvested mechanically and pelleted at 1,000 g. The cells

were then lysed in 400 µL of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 5 µg/ml aprotinin, 5 µg/ml pepstatin A, 5 µg/ml leupeptin and 0.1% Nonidet P-40) for 15 min on ice. The lysates were vortexed for 15 s and centrifuged at 16,000 g for 30 s. The pelleted nuclei were resuspended in 40 µl of buffer B (20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 0.12 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5 µg/ml aprotinin, and 5 µg/ml leupeptin). After 30 min on ice, lysates were centrifuged at 16,000 g for 10 min. Supernatants containing the nuclear proteins were diluted with 20 µl of buffer C (20 mM Hepes, pH 7.9, 20% (v/v) glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) and stored at -80° C. EMSA were performed using the NF-κB Binding Protein Detection System (Gibco BRL, Gaithersburg, MD) (20). Briefly, 10 µl of the nuclear extract (~ 1 µg) were mixed with 5 µl of 5X incubation buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 5 mM DTT, 5 mM EDTA 20% (v/v) glycerol and 0.4 mg/ml sonicated salmon sperm), with or without 100 fold excess of unlabeled probe and brought to a final volume of 25 µl with water. The mixture was incubated for 15 min at 4° C, and then 1 µl of radiolabeled (40,000 cpm) oligonucleotide specific for the NF-κB binding region was added and the mixture was incubated for another 20 min at room temperature. Bound oligonucleotide was resolved by electrophoresis of 15 µl of each sample (10,000 cpm) on a 6% nondenaturing polyacrylamide gel with TBE buffer (TRIS, Borate, EDTA). Gels were run for 90 min at 100 V at RT, dried, and then exposed to film overnight at -80 C.

7. Measurement of Cellular Viability by MTT assay

Cell viability was measured by the ability of the mitochondrial dehydrogenase to convert 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO) to an insoluable colored formazan. MTT was dissolved in water at a concentration of 5 mg/ml then syringe filtered through a 0.2 μm filter and stored at 4° C. After experimental treatment of cells the media was poured off and 50 μl of MTT stock solution in 450 μl of media was added to each well. The cells were then incubated at 37° C for 3-4 hrs. The media was discarded and the MTT salt was extracted with 500 μl/well of acidic isopropanol (0.1 N HCl in isopropanol) and the absorbance of the converted dye was measured at a wavelength of 570 nm with a reference wavelength of 620 nm using an automated spectrophotometric plate reader. Results are expressed as mean $\pm$ SE of absorbances.

8. Statistics

For all data where $n \geq 6$, comparisons between two groups were made using the Wilcoxon signed rank test (57). P-values of $< .05$ were considered statistically significant.

RESULTS

1. Stimulation of IL-8 elaboration by oxidative stress

The relationship between oxidative stress and IL-8 production in Beas-2b and MRC-5 cells was examined by stimulating the cells with media containing H_2O_2. Both Beas-2b and MRC-5 cells demonstrated H_2O_2 concentration dependent elaboration of IL-8 in response to H_2O_2 (Figure 7). Beas-2b cells stimulated with 0.5 mM H_2O_2 elaborated 176.3 ± 61.8 pg/ml of IL-8 compared to 9.9 ± 3.1 pg/ml IL-8 in control cells. Similar results were found in MRC-5 cells. When stimulated with 0.3 mM H_2O_2, MRC-5 cells elaborated 507 ± 45 pg/ml of IL-8 compared to 322 ± 40.1 pg/ml IL-8 in media from non-stimulated cells. H_2O_2 concentrations >0.5 μM in Beas-2b and >0.3 μM in MRC-5 cells were cytotoxic (Figure 8). These results demonstrate that oxidative stress and H_2O_2 in particular can stimulate IL-8 elaboration.

2. Rhinovirus induces oxidative stress in a bronchial epithelial cell line

The effect of hRV stimulation of IL-8 challenge on the oxidative state of the cell was examined with carbonyl and DCFDA in Beas-2b cells. MRC-5 cells were not used in these experiments because fibroblasts have a constitutively high level of oxidative stress that is attributed to a mechanism by which these cells stimulate growth (10,77).

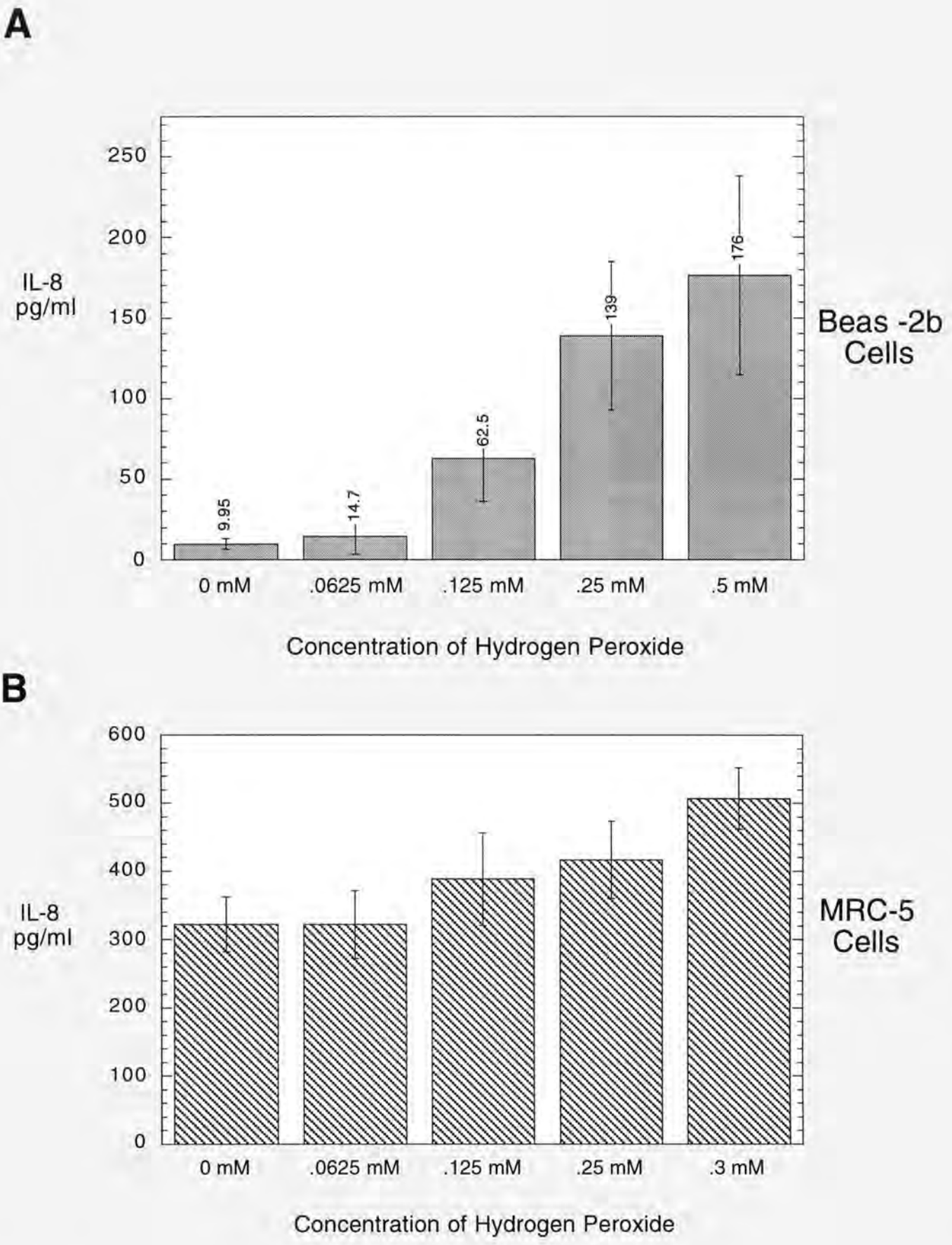

Fig. 7. ***Stimulation of IL-8 by hydrogen peroxide.*** Mean IL-8 concentrations (± SE) in supernatant media from Beas 2b cells (**A**) and MRC-5 cells (**B**) stimulated with increasing concentrations of H_2O_2. Supernatants were collected at 6 hrs.

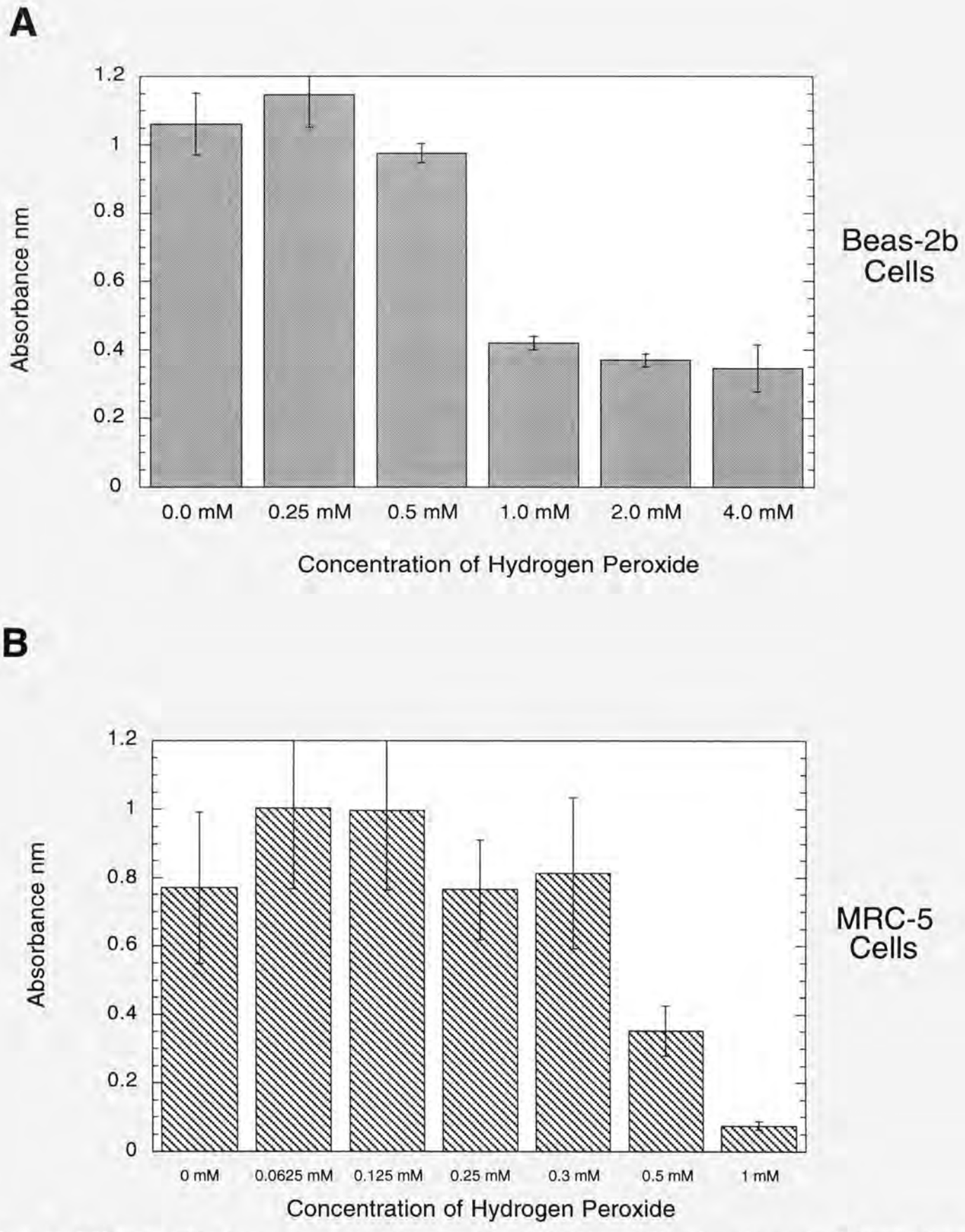

Fig. 8. ***Effects of hydrogen peroxide on cell viability.*** After treatment of Beas-2b cells (**A**) and MRC-5 cells (**B**) for 6 hrs with various concentrations of H_2O_2, cell viability was determined by MTT assay (mean ± SE).

A. Carbonyl staining

Cellular oxidative stress results in peroxidation of lipids and amino acids resulting in the formation of carbonyl groups. Quantification of ketone and aldehyde groups can thus be used as an indirect measure of oxidative stress. Detection of carbonyl groups by fluorescence microscopy showed increased formation of carbonyl groups in individual hRV infected Beas-2b cells following hRV challenge (Figure 9). Unchallenged Beas-2b cells had little carbonyl staining . Cells treated with 15% H_2O_2 for 15 min at 37° C were included as a positive control for the assay.

B. DCFDA staining

DCFDA is taken up by cells and produces a fluorescent product under conditions of oxidative stress. Beas-2b cells pretreated with purified virus for 6 hrs demonstrated an increase in fluorescent staining by DCFDA comparable to cells pretreated with 1 mM H_2O_2 for 2 hrs and greater than that of unstimulated cells treated with media alone (Figure 10). These results along with the carbonyl staining provide evidence that rhinovirus challenge of Beas-2b cells results in oxidative stress.

3. H_2O_2 production in response to hRV challenge

Because rhinovirus stimulated oxidative stress and H_2O_2 stimulated IL-8, we wanted to determine if H_2O_2 was one of the ROS stimulated by rhinovirus. The concentration of H_2O_2 elaborated into supernatant media was measured to assess

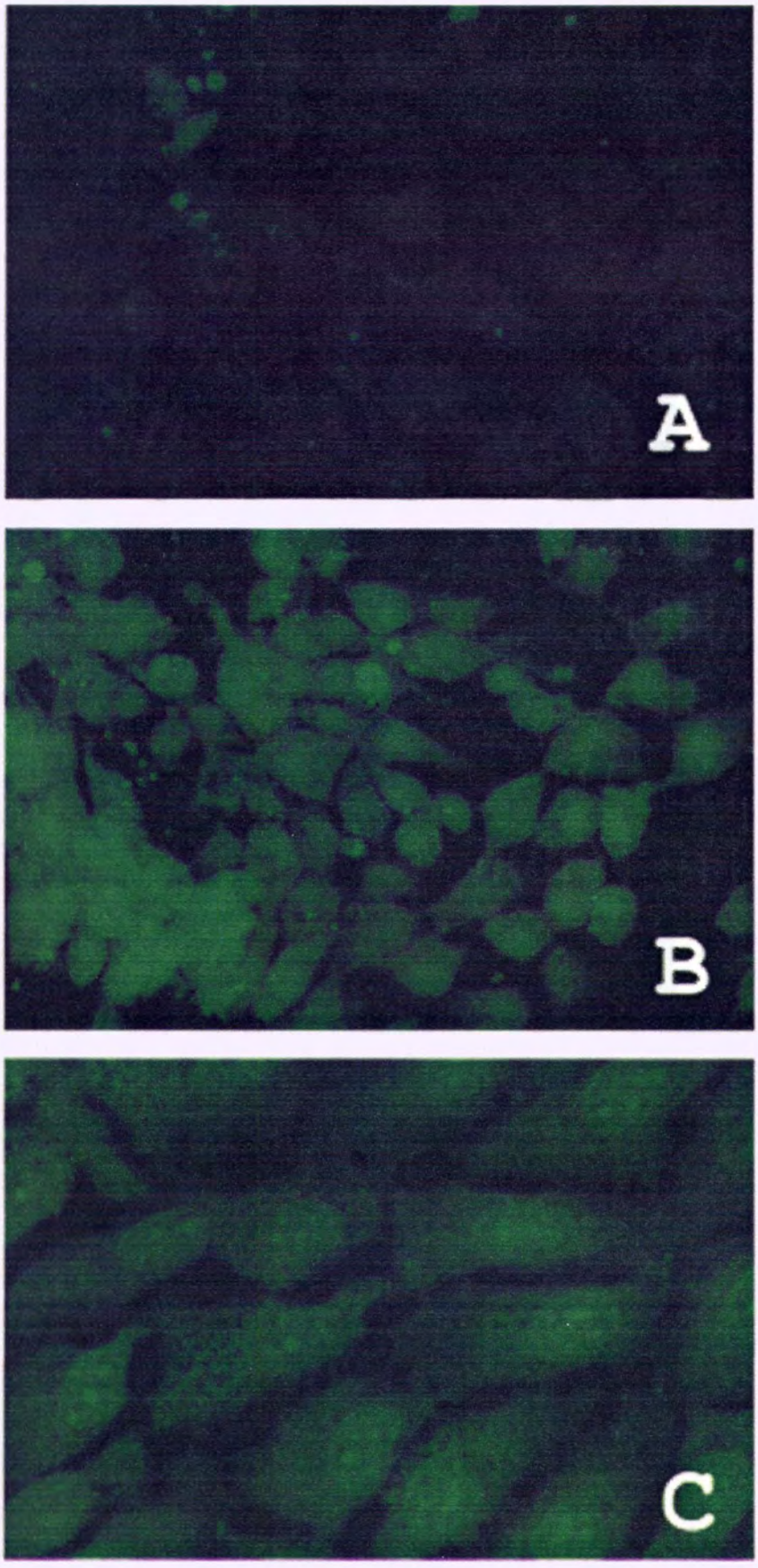

Figure 9. Carbonyl staining in vivo of hRV and oxidant treated cells. Untreated cells had almost no carbonyl staining (A). Cells treated with 15% H2O2 demonstrated the greatest amout of fluorescence (B), with a greater ammount of staining in the perinuclear region. Viral treated cells (C) demonstrated the highest amount of staining in the nuclear region, including both the nuclear membrane as well as spherical bodies with in the nucleus itself.

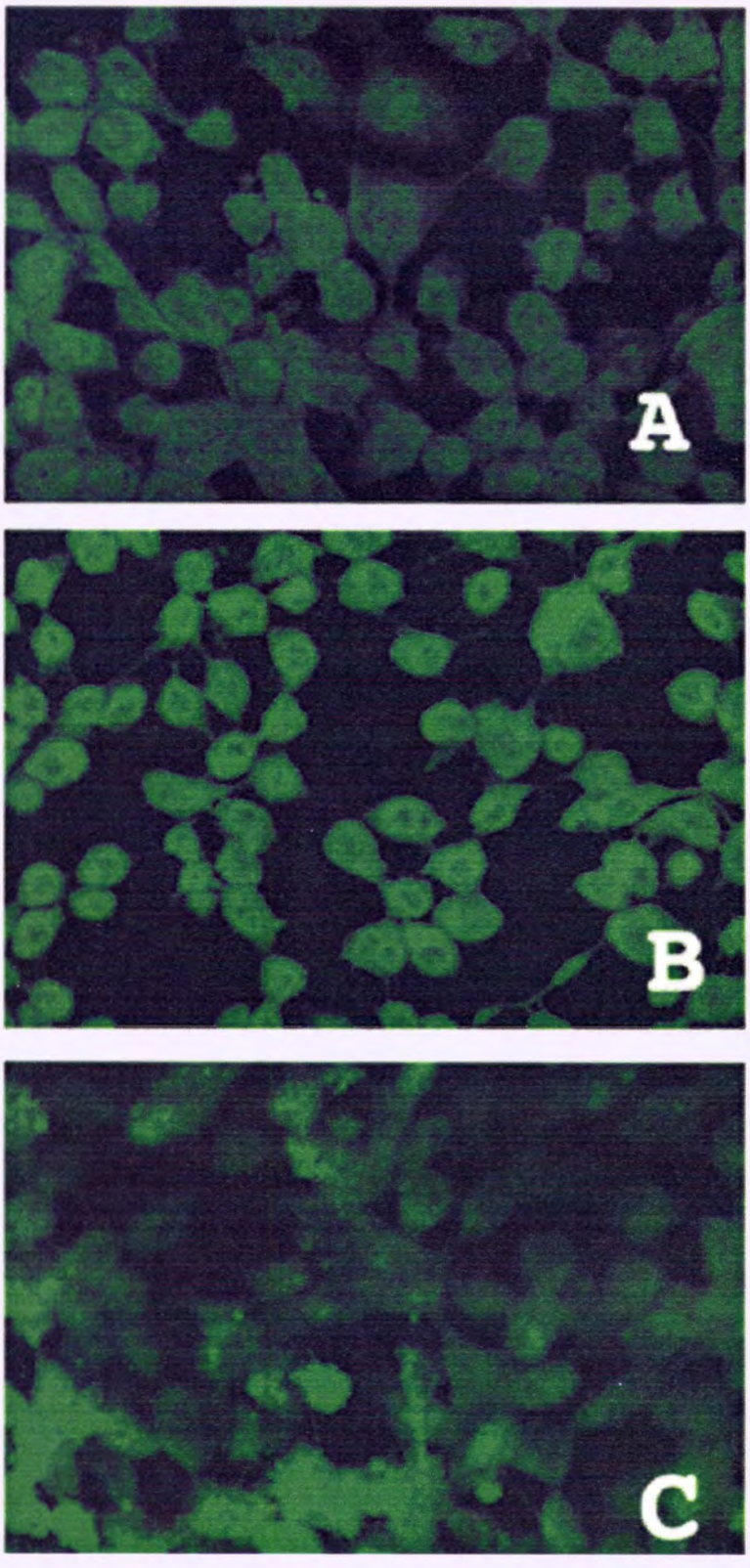

Figure 10. DCFDA staining of H2O2 and hRV stimulated cells in vivo. Beas-2b cells demonstrated an increase in fluorescent staining by by DCFDA after inoculation with virus (C) comperable to cells pretreated with 1mM H2O2 for 3 hrs (B) and greater than that of unstimulated cells treated with media alone (A).

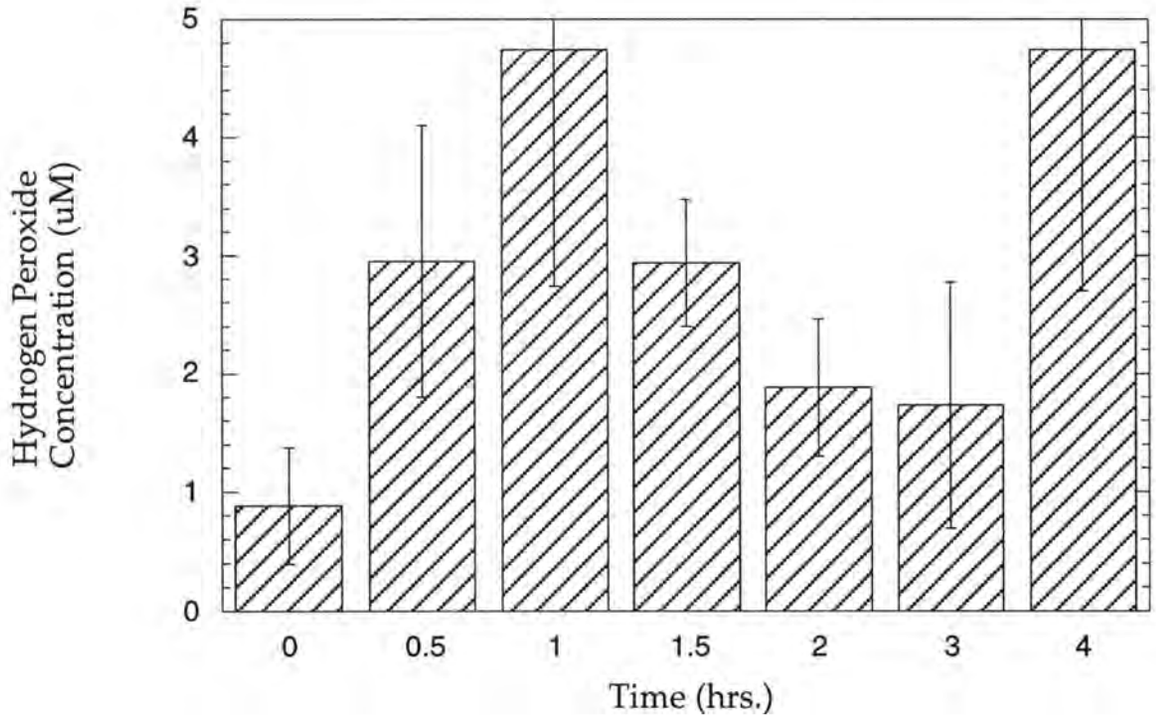

Fig. 11. ***Time dependent hydrogen peroxide concentration in media supernatant from Beas-2b cells challenged with hRV.*** The H_2O_2 concentration in media collected 4 hours after virus challenge was significantly greater ($p<0.01$, Wilcoxon signed rank test) than in sham challenged control cells.

H_2O_2 production in response to hRV challenge. Again, MRC-5 cells were not examined for an H_2O_2 response because of their constiutively high level of oxidative stress as described above. Media supernatants from Beas-2b cells challenged with hRV contained H_2O_2 concentrations of 4.74 ± 2.4 μM compared to 0.4 ± 0.49 μM ($p<0.01$, Wilcoxon sign rank method) in unchallenged cells 4 hrs after stimulation (Figure 11). These results were verified with purified hRV (data not shown). The H_2O_2 response was first noted 30 min after virus challenge and reached a maximum by 1 hr. Limited data at the 1.5, 2, and 3 hr time points suggest a biphasic H_2O_2 response, but this was not confirmed. These results suggest that hRV induced oxidative stress may mediate up regulation of IL-8.

4. Effect of antioxidants on hRV stimulation of IL-8 in respiratory epithelial cell lines.

Because oxidative stress can stimulate IL-8 and infection of Beas-2b cells with hRV causes oxidative stress, the effects of several different antioxidants on hRV induction of IL-8 were examined. MRC-5 and Beas-2b cells were treated with five different antioxidants; NAC, DMSO, PBN, DMPO and ferulic acid, and hRV stimulated IL-8 elaboration was measured.

A. N-acetyl cysteine (NAC) Inhibits IL-8 production by purified hRV in vitro

Beas-2b cells challenged with hRV produced 35.8 ± 2.1 pg/ml compared to 3.5 ± 0.0 pg/ml of IL-8 elaborated by unstimulated cells (Figure 12). NAC inhibited IL-8 production by virus stimulated cells. In the presence of 30 mM NAC, the highest concentration tested, virus stimulated Beas-2b cells produced 10.5 ± 0.9 pg/ml of IL-8, a 70% reduction compared to virus challenged cells without NAC treatment.

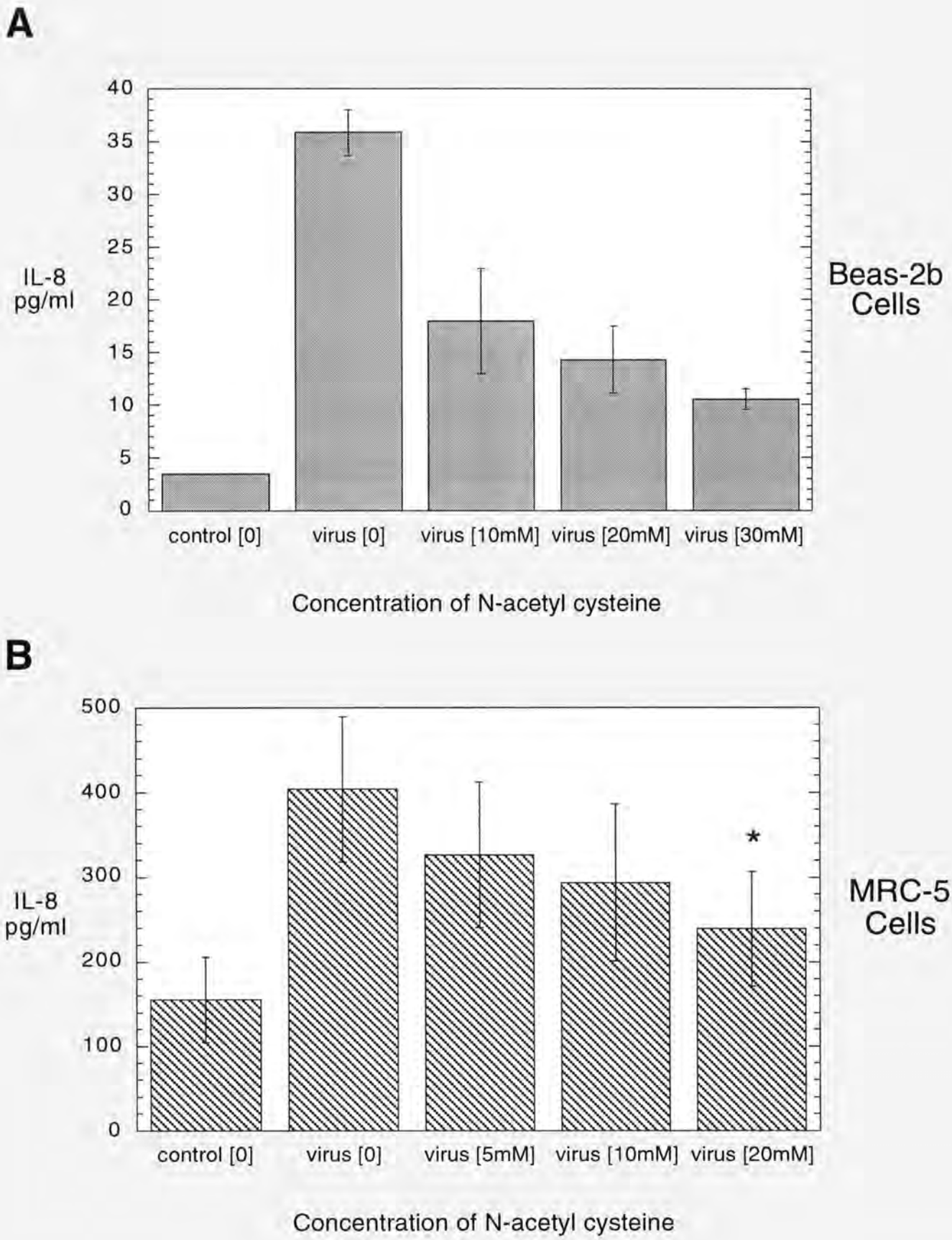

Fig. 12. ***Inhibition of hRV induced IL-8 by N-acetyl cysteine***. Both Beas-2b (**A**) and MRC-5 (**B**) cells were treated with NAC for 1 hr prior to virus challenge. IL-8 concentrations were measured in media supernatants collected 6 hrs after challenge. (* = $p<0.02$, Wilcoxon signed rank test).

NAC also produced a dose dependent inhibition of viral induced IL-8 in MRC-5 cells, with viral stimulated cells producing 403.8 ± 85.49 pg/ml of IL-8 compared to 239± 67.7 pg/ml of IL-8 elaborated by viral stimulated cells in the presence of 20 mM NAC ($p < 0.02$, Wilcoxon sign method). None of the NAC concentrations used caused cytotoxicity in either cell line as measured by the MTT assay (Figure 17).

B. Inhibition of IL-8 with purified hRV in DMSO treated cells in vitro

Pretreatment of Beas-2b and MRC-5 cells with DMSO inhibited hRV induced IL-8 production (Figure 13). Beas-2b cells treated with 3% DMSO elaborated 15.4 ± 6.1 pg/ml IL-8 compared to 112.7 ± 5.3 pg/ml IL-8 elaborated by control cells after stimulation with hRV. Similarly, MRC-5 cells treated with 2% DMSO reduced IL-8 production to 94.1 ± 19.8 pg/ml upon viral stimulation compared to 217.5 ± 71 pg/ml elaborated by untreated viral stimulated controls cells. The DMSO concentrations used did not cause cytotoxicity in either cell line as measured by the MTT assay (Figure 17).

C. Stimulation of IL-8 with purified hRV in PBN and DMPO treated cells in vitro

Pretreatment of Beas-2b cells with DMPO or PBN had no measurable effect on viral induced IL-8 elaboration (Figures 14 & 15). Beas-2b cells treated with 500 µM DMPO and challenged with hRV elaborated 83.6 ± 15 pg/ml as compared to virally challenged unstimulated cells which produced 112 ± 5.5 pg/ml. Beas-2b cells treated with 200 µg/ml of PBN and stimulated with virus produced 83.5 ± 35 pg/ml compared to viral controls which elaborated 86.8 ± 25 pg/ml. Pretreatment of MRC-5 cells with either DMPO or PBN showed no detectable effect upon the elaboration of IL-8 in hRV stimulated cells at 6 hrs. MRC-5 cells treated with 500

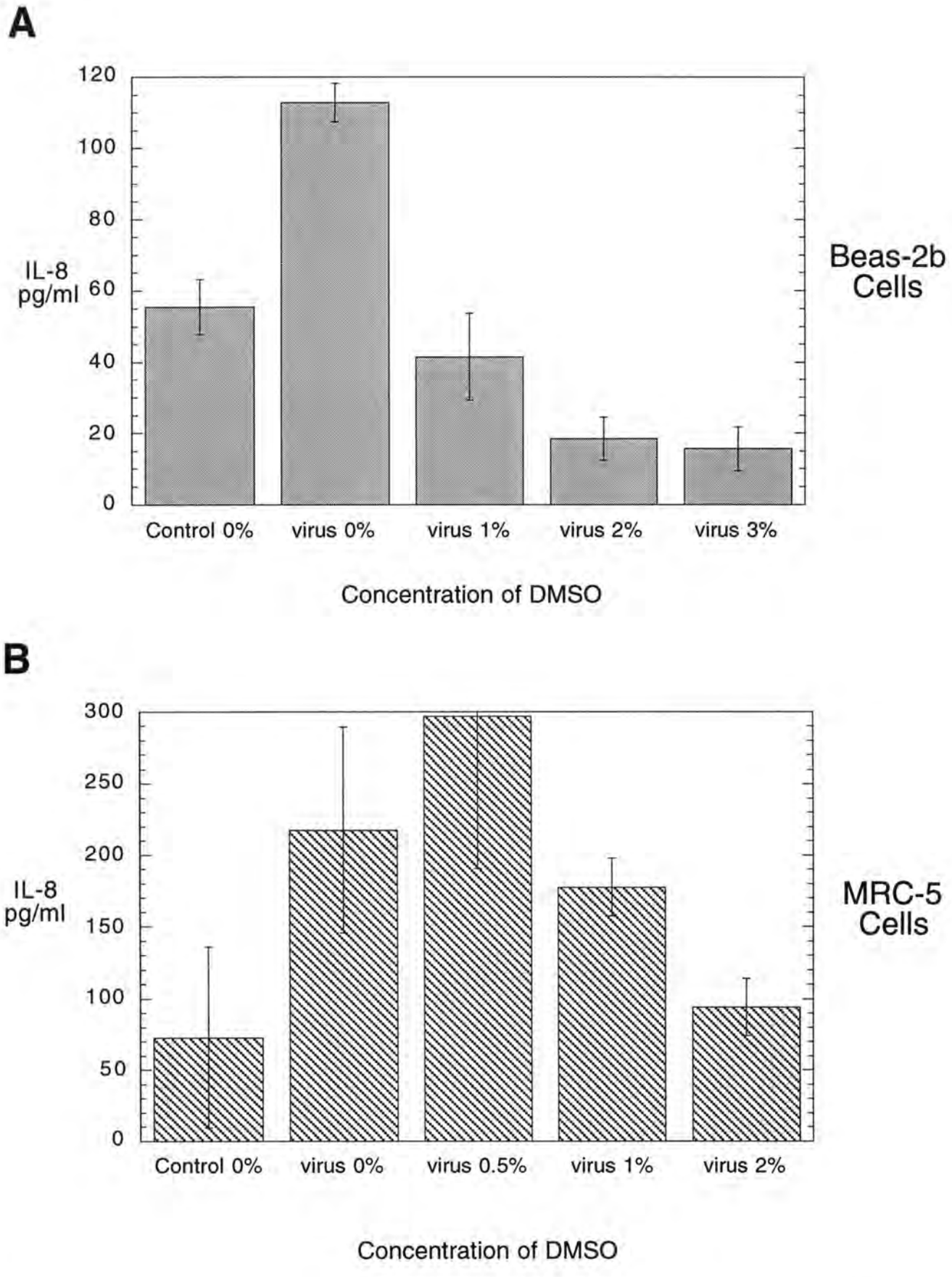

Fig. 13. ***Inhibition of hRV induced IL-8 by DMSO.*** Beas-2b (**A**) and MRC-5 (**B**) cells were pretreated with DMSO 1 hr prior to virus challenge. IL-8 (mean ± SE) concentrations were measured in media supernatants 6 hrs after virus challenge.

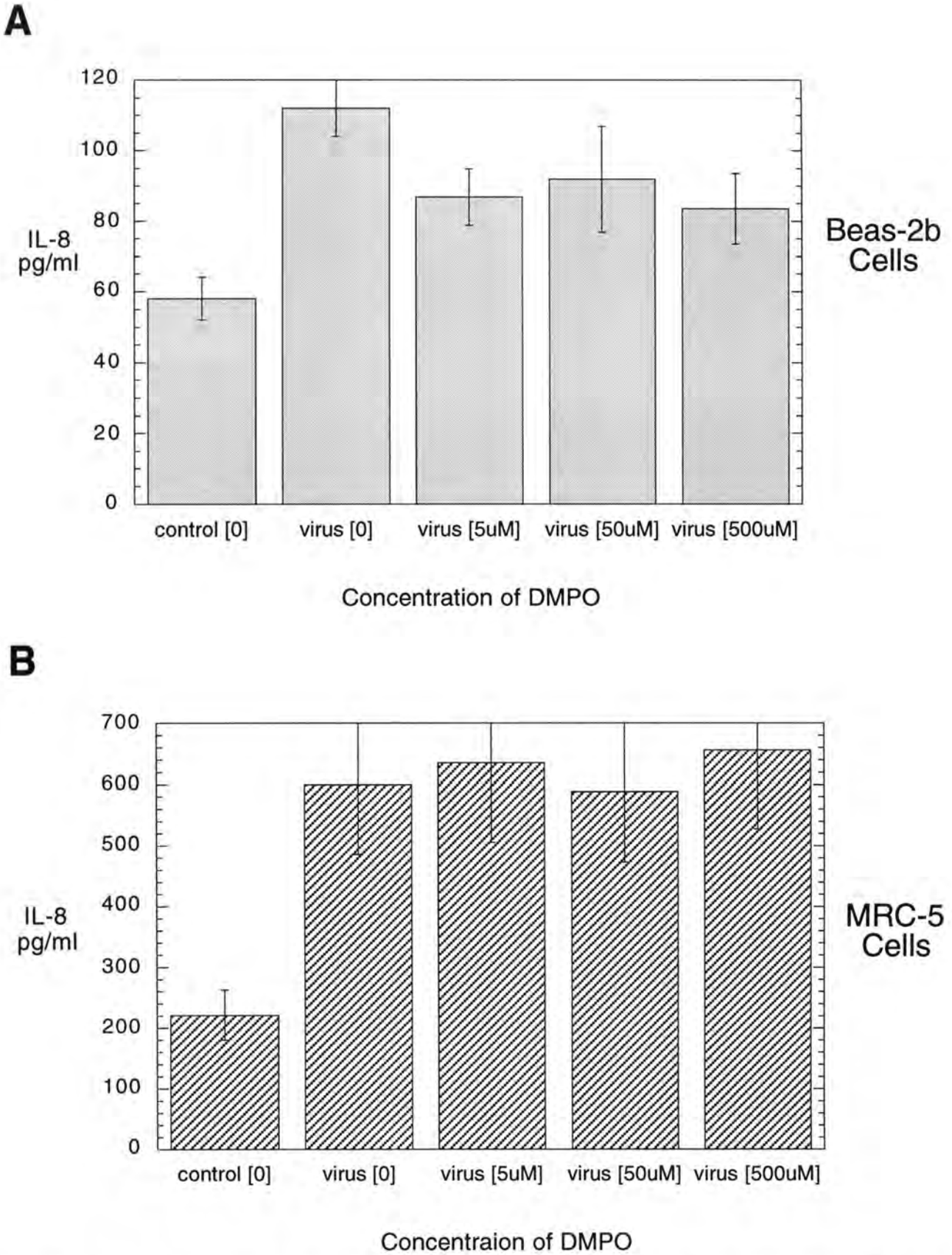

Fig. 14. ***Effects of DMPO on hRV stimulation of IL-8.*** Beas-2b (**A**) and MRC-5 (**B**) cells were pretreated with DMPO for 1.5 hrs prior to challenge with hRV. IL-8 (mean ± SE) was measured from supernatants collected 6 hrs after virus challenge.

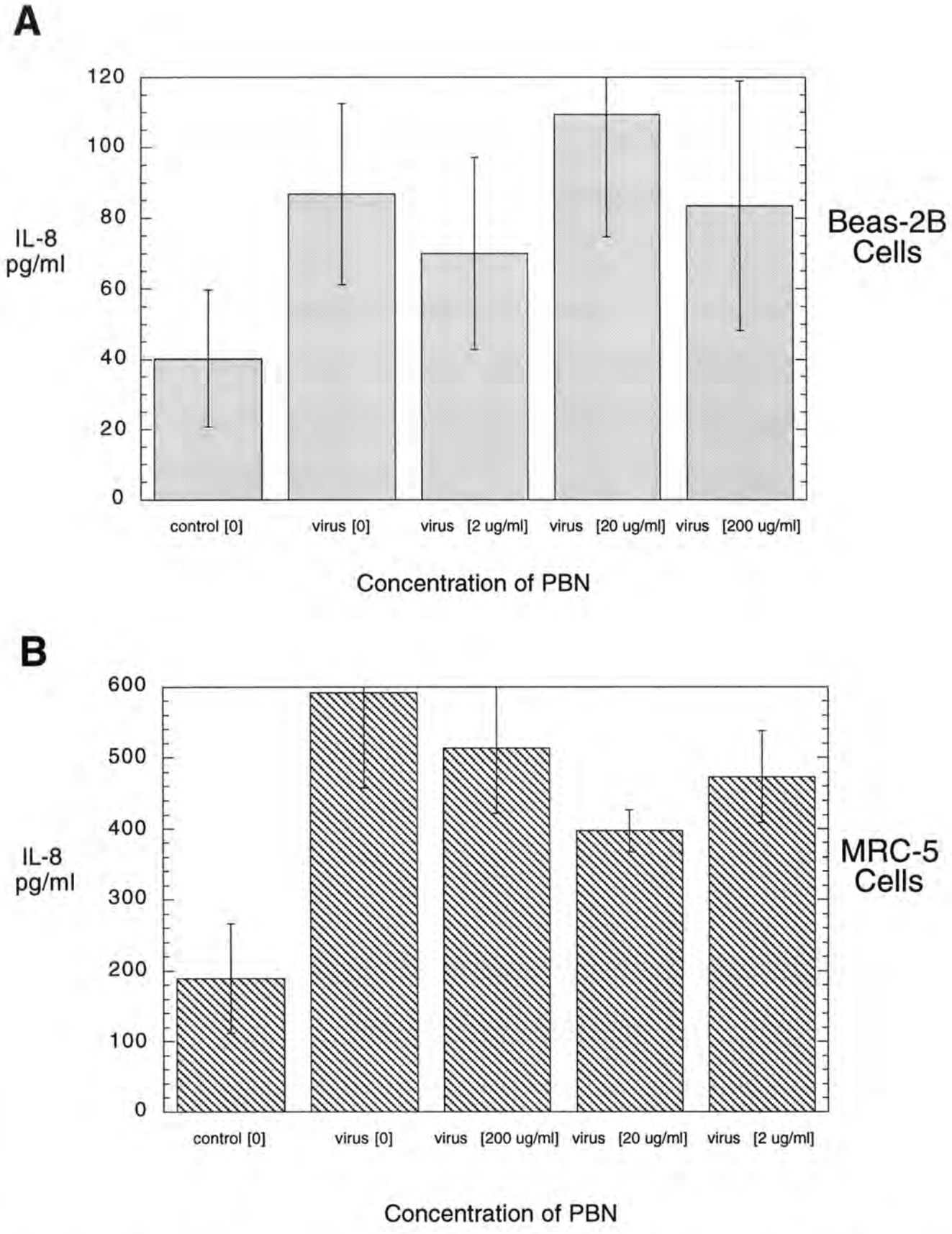

Fig. 15. ***Effects of PBN on hRV induced IL-8.*** Beas-2b (**A**) and MRC-5 (**B**) cells were pretreated with PBN for 1.5 hrs prior to virus challenge. IL-8 (mean ± SE) was measured from supernatants collected 6 hrs after virus challenge.

μM DMPO and challenged with hRV elaborated 656.5 ± 128 pg/ml as compared to virally challenged untreated cells which produced 599.0 ± 114 pg/ml. MRC-5 cells treated with 200 μg/ml of PBN and stimulated with virus produced 473.0 ± 64 pg/ml compared to viral controls which elaborated 592.2± 134 pg/ml.

D. Stimulation of IL-8 with purified hRV in ferulic acid-treated cells in vitro

In Beas-2b cells, ferulic acid at a concentration of 5 mM inhibited viral induced IL-8 by 39% with viral stimulated IL-8 dropping from 119.5 ± 0.9 pg/ml to 73.1 ± 2.0 pg/ml upon pretreatment with 5 mM ferulic acid. HRV stimulated MRC-5 cells demonstrated no dose dependent response to treatment with the antioxidant ferulic acid. Viral stimulated cells pretreated with 2.5 mM ferulate produced 515.2 ± 24.2 pg/ml IL-8 as compared to virally stimulated untreated cells which elaborated 632.0 ± 26.9 pg/ml IL-8 at the same 6 hr time course (Figure 16). None of the ferulate concentrations used produced cytotoxic effects in either cell line (Figure 13). These data show that ferulic acid inhibits IL-8 stimulated by hRV in Beas-2b cells but not MRC-5 cells.

5. NF-κB EMSA in H_2O_2 and purified viral challenged cells treated and untreated with NAC in vitro

NF-κB activation appears to be the main mechanism by which cells up-regulate IL-8 transcription. In order to further investigate the relationship between hRV induction of IL-8 and oxidative stress, the effects of both H_2O_2 and hRV on NF-kB were examined. In addition, in light of the inhibitory effects of NAC and DMSO on hRV induced IL-8 elaboration, the effect of these agents on NF-κB activation by hRV was also evaluated. Experiments were performed only in Beas-2b cells because MRC-5 cells have a high level of NF-kB activation under basal conditions.

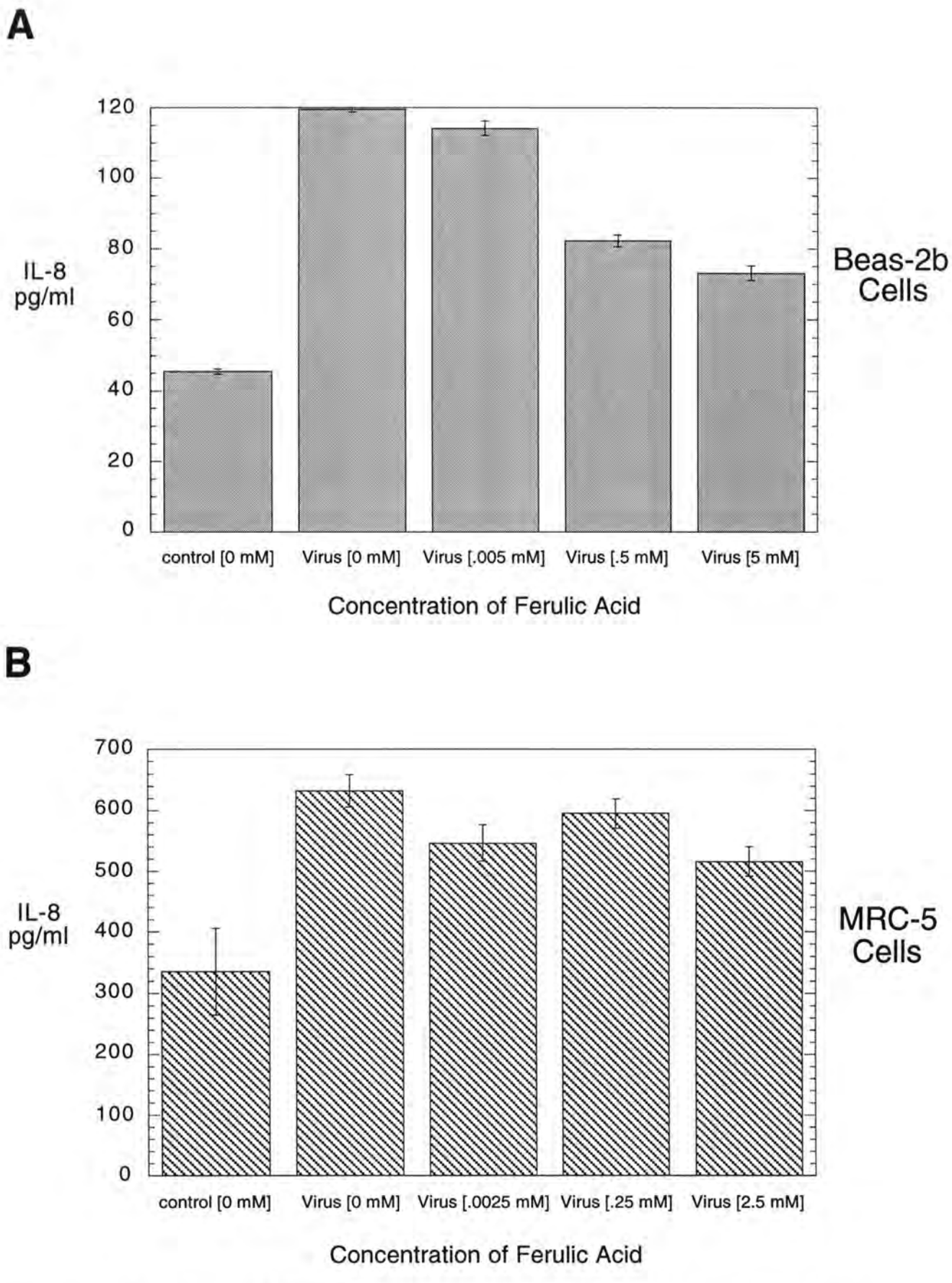

Fig. 16. ***Inhibition of hRV induced IL-8 by ferulic acid.*** Beas-2b (**A**) and MRC-5 (**B**) cells were pretreated with ferulate for 1.5 hrs prior to challenge with hRV. IL-8 (mean ± SE) was measured from supernatants collected 6 hrs after virus challenge.

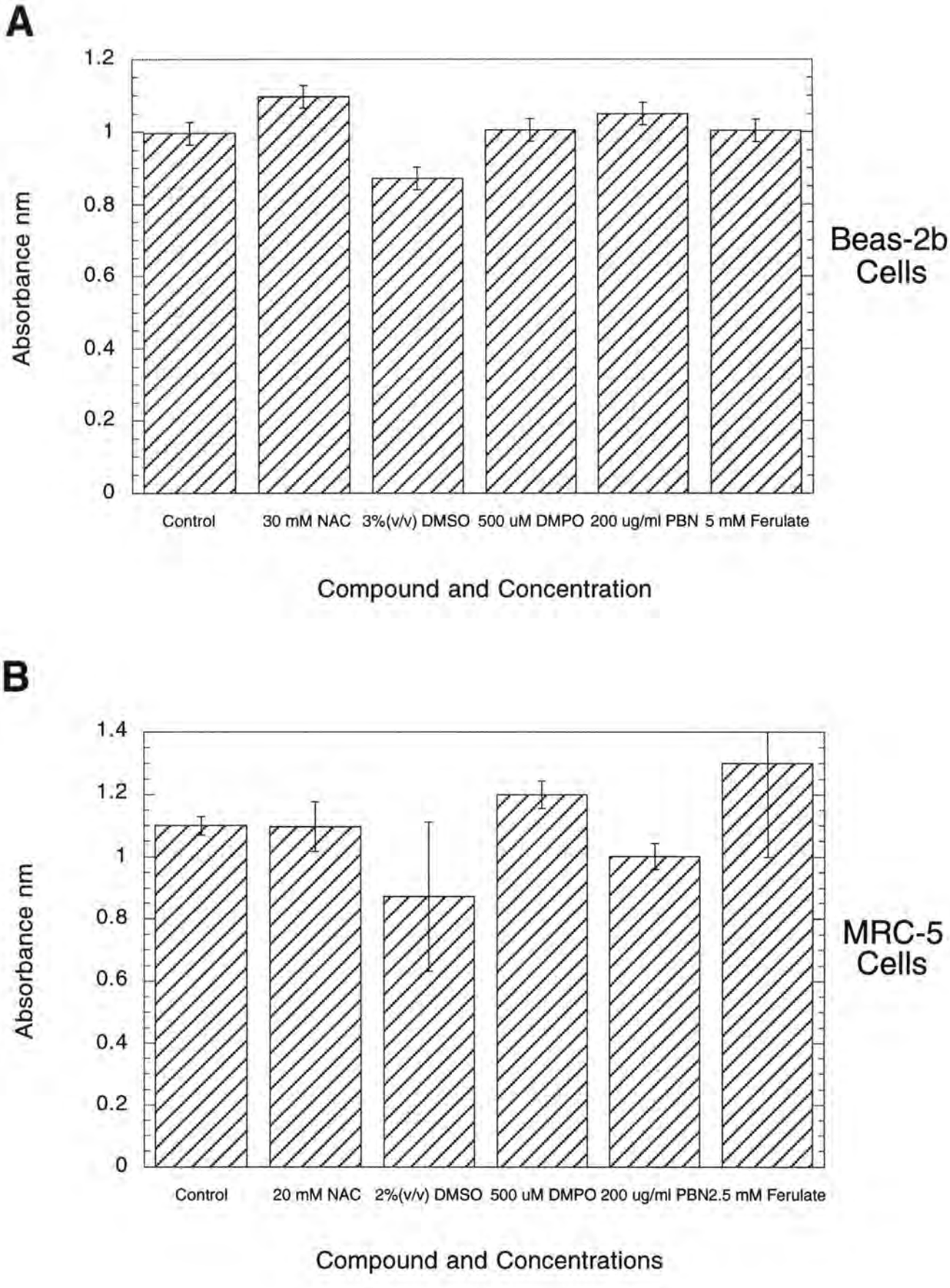

Fig 17. ***Effects of various antioxidants on cell viability.*** Cell viability was determined by MTT assay in Beas-2b (**A**) and MRC-5 (**B**) cells collected after 8 hrs of exposure to various antioxidants.

Treatment of Beas-2b cells with H_2O_2 or challenge of cells with hRV both strongly activated NF-κB in Beas-2b cells (Figure 18). These shifts were attenuated by the addition of excess unlabeled competitor probe demonstrating protein/nucleotide binding specificity. Pretreatment of cells with NAC (20mM) 2 hrs prior to viral exposure, inhibited hRV's ability to translocate NF-κB. However, pretreatment of Beas-2b cells with media containing 3% DMSO for 2 hrs did not inhibit hRV activation of NF-κB (Figure 19). Cells treated with 20 mM NAC or 3% DMSO without virus challenge demonstrated no increased activation of NF-κB (Figure 20) as compared to untreated cells. These results provide evidence that both hRV and H_2O_2 activate NF-κB and that hRV activation of NF-kB can be inhibited by the antioxidant NAC but not DMSO.

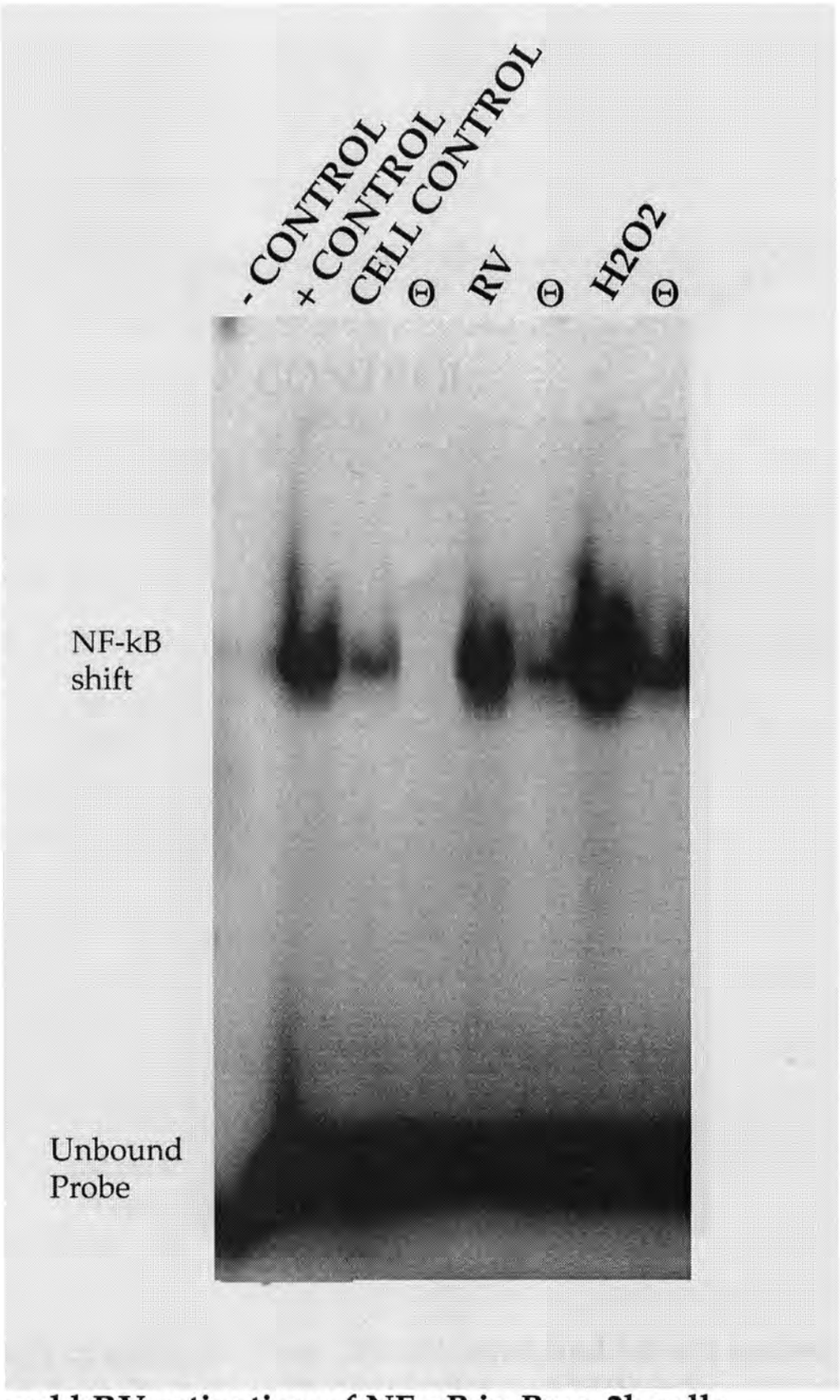

Fig.18. **H2O2 and hRV activation of NF-κB in Beas-2b cells.**
2 hr treatment of Beas-2b cells with either 1mM H_2O_2 or a viral MOI of a 100 $TCID_{50}$ resulted in translocation of NF-κB to the nucleus. (- Control is labeled probe by itself and + Control is HeLa nuclear extracts after pretreatment with PMA). The top band represents specific NF-κB complexes, where as the lower band is unbound oligonuclotide. Θ represents addition of 100X excess of unlabelled probe to condition to its left.

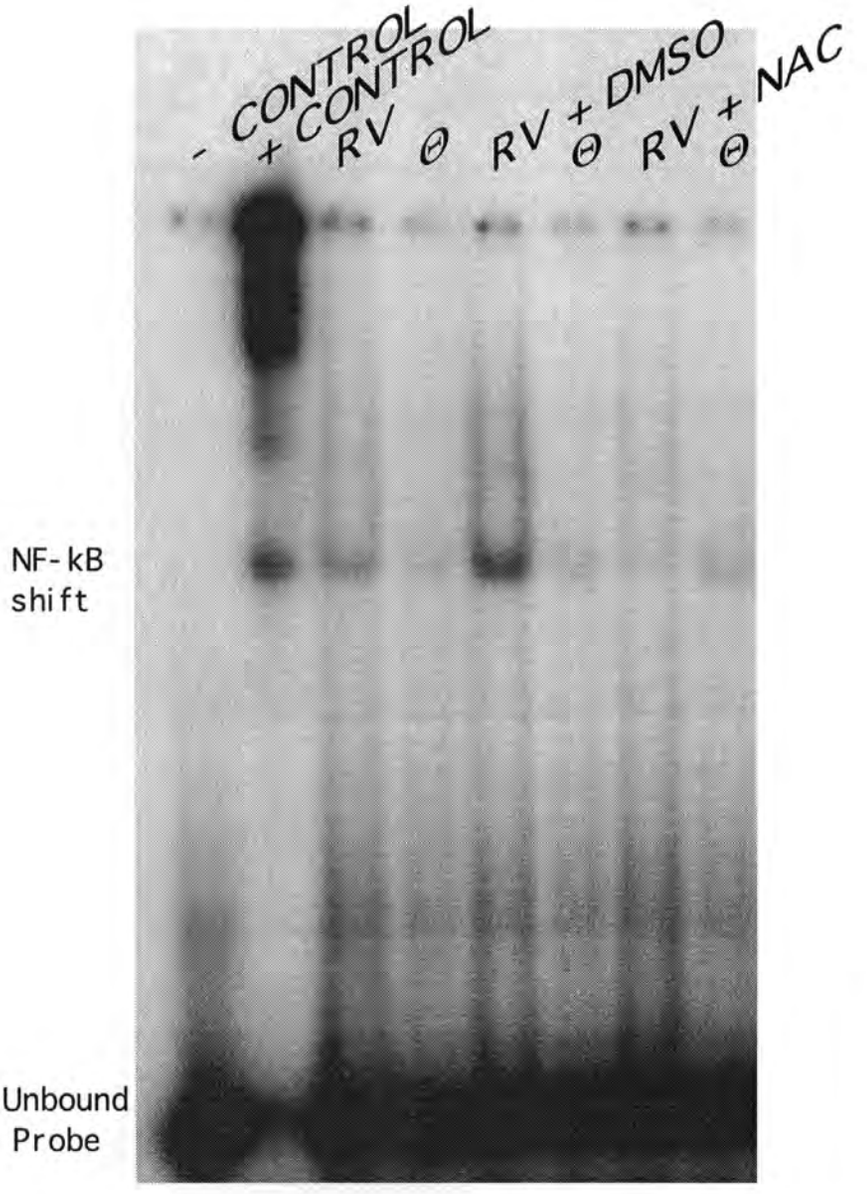

Fig. 19. **N-Acetyl cysteine but not DMSO inhibited NF-κB activation**. 2 hr pretreatment of Beas-2b cells with NAC inhibited hRV induction of NF-κB activation. Identical treatment of cells with DMSO resulted in no change of hRV activation of NF-κB (- and + Controls are the same as in Fig. 18). The top band represents specific NF-κB complexes, where as the lower band is unbound oligonuclotide. Θ represents addition of 100X excess of unlabelled probe.

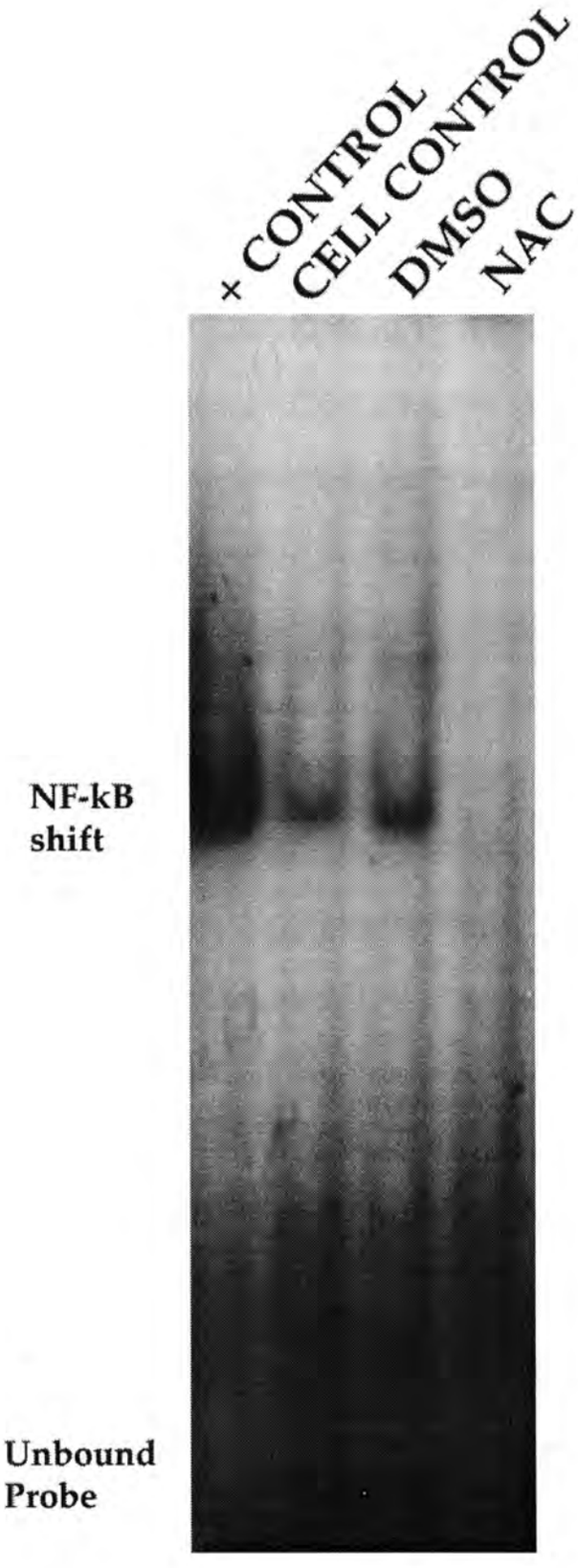

Fig. 20. **N-Acetyl cysteine and DMSO do not increase NF-κB activation**. 2 hr pretreatment of Beas-2b cells with NAC or DMSO resulted in no increase of activation of NF-κB (+ Control is the same as in Fig. 18). The top band represents specific NF-κB complexes, where as the lower band is unbound oligonuclotide.

Discussion

Although involvement of IL-8 as a mediator of rhinovirus colds is generally accepted, very little information has been generated regarding the mechanism by which hRV induces IL-8. The purpose of this study was to determine if reactive oxygen species are involved in up-regulation of IL-8 by hRV. Our studies demonstrate that rhinovirus infection of a human respiratory epithelial cell line results in increased production of hydrogen peroxide and oxidative stress. Our studies also show that the addition of exogenous ROS will up-regulate IL-8 in two unrelated respiratory cell lines. In addition, it was found that treatment of respiratory cell lines with the antioxidants NAC, DMSO and ferulic acid inhibited hRV induced IL-8. Lastly, our findings demonstrate that, in Beas-2b cells, both ROS and hRV cause the activation of NF-κB and that this NF-κB activation by hRV is inhibited by the antioxidant NAC but not DMSO.

Three separate techniques were employed to demonstrate viral induction of oxidative stress. Although in the past, there have been difficulties in correlating oxidation with changes invoked in diseased cells, it has been found that increases in carbonyl staining is a reliable indicator of oxidative stress (32). Oxidative processes, including peroxidation of lipids and amino acids, generate a number of by-products (32). Of these products, aldehydes are among the most prevalent and can be detected by the binding of carbonyls to fluorescein labeled hydrazides. Using this method we were able to demonstrate that infection of respiratory epithelial cells with hRV causes oxidative stress on the cells. The

presence of viral induced oxidative stress was confirmed by staining infected cells with DCFDA. DCFDA is actively transported into the cell and, in the process, the acetate groups are removed by membrane esterases, resulting in dichlorofluorescein (DCF) which is can not cross the plasma membrane as easily as the acetylated form (8). DCF, which is nonfluorescing, becomes dechlorinated upon oxidation, resulting in the fluorescent compound fluorescein. Therefore, the amount of DCFDA converted to fluorescein reflects the oxidative state of the cell and is a good indicator of oxidative stress. In addition to visually confirming hRV induced oxidative stress by fluorescence, we were able to use a quantitative technique established by Pick et al. (1981) to measure increases in H_2O_2 concentrations in the supernatant of hRV infected Beas-2b cells as compared to noninfected cells (52). Oxidative stress was not examined in MRC-5 cells because fibroblast cell lines have a constiutively high level of oxidative stress (10,77). It is believed that, in MRC-5 cells, oxygen radicals might trigger an adaptive stress response which remove damaged macromolecules and thereby increase the cells' growth potential (26).

Our study demonstrates that treatment with H_2O_2 causes an increase in the release of IL-8 in both MRC-5 and Beas-2b cells. Even though oxidative tone has been suggested to be involved in both LPS and respiratory syncytial virus induced IL-8, this is the first time ROS have been shown to directly up-regulate IL-8 elaboration in either lung fibroblasts or a bronchial epithelial cell line(37,13). The discrepancy between the amount of H_2O_2 measured in rhinoviral infected cell supernatants (5 μM) and the amount of exogenously added H_2O_2 required to stimulate IL-8 (5 mM) can be explained as follows; Although there is a 3 log difference in H_2O_2 concentrations, it should be recognized that the amount of H_2O_2 added exogenously does not necessarily reflect the intracellular

concentration. Also, the converse stands true. The amount of H_2O_2 produced intracellulary is not accurately reflected by the amount of H_2O_2 measured extracellularly. Two main factors that account for this discrepancy are a) the cell's inherent ability to reduce oxidative stress through enzymatic reduction and b) H_2O_2's inability to cross the plasma membrane with perfect efficiency. Despite this discrepancy, the observation that epithelial cells produce ROS upon infection with hRV and that ROS, by themselves, can cause IL-8 elaboration, suggests that ROS may act as a mediator of hRV induced IL-8.

If hRV stimulated IL-8 is mediated by oxidative stress, it would be expected that the process could be inhibited by antioxidants. Because antioxidants work in different fashions, five different antioxidants effects on hRV stimulated IL-8 elaboration were examined.

N-acetyl cysteine is a cysteinyl thiol that can directly except 2 e- to form NAC thiyl radical intermediate (35). NAC easily crosses membranes because of its acetyl group and has the ability to become deacetylated, thereby supporting glutathione (GSH) synthesis (78,35). GSH is available through out the cytoplasm and is able to scavenge a variety of ROS including phenoxyl, peroxyl, semiquinone, carbon centered, and hydroxyl radicals. These adducts are quickly reduced and metabolized through the action of several enzymes, including glutathione peroxidase and glutathione-S-transferase (35).

DMSO can capture $OH^{\bullet}$ radicals to form methanesulphinic acid (MSA) and a much less reactive radical as shown bellow:

$$H_3C\text{-}SO\text{-}CH_3 + OH^{\bullet} \longrightarrow {}^{\bullet}CH_3 + H_3C\text{-}SO\text{-}OH$$

DMSO MSA

This reaction has an 85% yield rate (1). The •CH3 radical may further change to methane ethylene or formaldehyde. The hydroxyl radical, OH•, is extremely reactive with most compounds with a rate constant around 7E9 M^{-1} s^{-1} (64). DMSO can easily cross cellular membranes and is readily available throughout the cell, making it a very effective antioxidant.

DMPO and PBN are two similarly structured nitrones that have demonstrated an ability to form adducts with superoxide, the hydroxyl radical, as well as a number of other oxygen, carbon, and nitrogen radicals (6, 5, 68). In addition, they have been shown to interfere with the dismutation of superoxide to H_2O_2 by serving as a competitive inhibitor (6). Although their most common application has been with spin trapping techniques in conjunction with electron paramagnetic resonance spectroscopy (EPR), they have proven efficacious as oxidative stress inhibitors in animal models, including stroke, aging, sepsis, and myocardial ischemia/reperfusion injury (68). Our purpose for including both compounds in this study and the major difference between DMPO and PBN are their hydrophobicities. PBN prefers the aqueous phase where as DMPO is much more lipophilic (68).

Ferulate is a cinnamonic acid derivative that scavenges hydroxyl ions as well as peroxides (RO2). Outside of its antioxidant capabilities it is thought to be relatively inert. Ferulate's structure lends itself towards the aqueous phase which allows it to be available throughout the cytoplasm.

Supporting the theory that ROS mediate hRV's IL-8 up-regulation, we found that hRV induced IL-8 could be inhibited by antioxidants. NAC, DMSO, and ferulic acid proved the most effective antioxidants in inhibiting IL-8 production.

PBN and DMPO had no measurable effect on hRV induced IL-8 elaboration. An explanation for this discrepancy could be that each of these antioxidants may be available in only certain compartments of the cell, not necessarily where oxidative stress is occurring (68). Therefore, it is possible that DMPO and PBN are not available in the location where there is oxidative stress. Another possibility as to why we did not observe inhibition with DMPO and PBN, is that the concentrations necessary to effectively prevent oxidative stress are either cytotoxic or insoluble. For both compounds we had solubility problems with our buffer system at concentrations above the uM range, where as most work done with PBN and DMPO is at concentrations greater than 10-100 mM (6). As opposed to DMPO and PBN, the other three antioxidants studied demonstrate a consistent inhibition of hRV induced IL-8 between the two different cell lines except for ferulic acid which demonstrated an inhibitory effect only in Beas-2b cells. Ferulic acid's inability to effectively inhibit hRV induced IL-8 elaboration in MRC-5 cells could be for similar reasons as to why DMPO and PBN were ineffective.

NAC, DMSO and ferulate, though antioxidants, are structurally dissimilar and work by different mechanisms. Because these antioxidants are so different, suggests that their means of hRV induced IL-8 inhibition is through their antioxidant capabilities and not some other cellular effect.

These results show that ROS are implicated as the mediator of hRV induced IL-8, and that their involvement is, in part, through the activation of the transcription factor NF-kB. Schreck et al. (1991) proposed that ROS act as a universal mediator for NF-κB translocation to the nucleus (60). Brennen and O'Neil (1995), however, have demonstrated that ROS mediation of NF-κB is not universally true, but rather, a cell type specific phenomenon (4). It was found that both hRV and hydrogen peroxide both effectively activated the transcription

factor, NF-κB, in Beas-2b cells. Moreover, it was found that NAC inhibited this activation. These observations suggest that oxidative stress, whether exogenously added or induced by viral infection, stimulate IL-8 production through the activation of NF-κB.

Inconsistent with this hypothesis, is the finding that DMSO did not inhibit NF-κB activation. However, DMSO has multiple effects on the cell that might explain this phenomenon. DMSO causes an increase in membrane permeability and, in some instances, can denature certain proteins such as lysozyme and certain ribonucleases (29). It is possible that DMSO inactivates some protein involved in antioxidant inhibition of NF-κB activation. A more compelling argument revolves around the fact that DMSO causes the release of intracellular Ca^{++} stores (36,7). Recently, Heike et al. (1996) have shown that oxidative stress induced NF-κB activation requires the involvement of Ca^{++} flux into the cytoplasm from intracellular stores (24). In addition, chealation of intracellular Ca^{++} was shown to inhibit H_2O_2 activation of NF-κB (24). If antioxidants were to inhibit NF-κB activation by the prevention of Ca^{++} release, then treatment with DMSO would be ineffective in blocking NF-κB activation because it causes the release of Ca^{++} on its own. This hypothesis suggests that DMSO inhibits IL-8 production through one of the other important transcription factors, NF-IL6 or AP-1, of which AP-1 has already been shown to be redox sensitive (56). In any case, it is reasonable to infer that hRV induced oxidative stress activates NF-κB which, in part, mediates hRV up-regulation of IL-8 gene transcription. The implications of these findings are that antioxidants in general and NAC in particular may prove to be effective treatments for hRV cold symptoms.

This study brings up several questions. The first question is if hRV activation of NF-kB is associated with any fluxes in Ca^{++}. Because DMSO does not

inhibit NF-κB activation by virus and that most of the known enzymes involved in the degradation of I-κB are Ca^{++} dependent, would suggest Ca^{++} involvement (24). Secondly, this study leads to the investigation of how and where hRV induces generation of oxidative species with in the cell. In our lab, we have shown that infection of Beas-2b cells leads to an increased activity of Cu/Zn superoxide dismutase which suggests an increased presence of superoxide (unpublished data). Therefore it is feasible that hRV may act to uncouple cellular respiration such as TNF-α at the mitochondrial level (63) leading to ROS generation or it may be through a novel mechanism as yet to be determined.

A major question to be addressed in future studies, however, is whether NAC can inhibit hRV induced IL-8 *in vivo* and in-turn common cold symptoms. NAC has been used clinically for many years. The primary medical applications of NAC have been for acetominophin intoxication as well as for a mucolytic purposes. These activities of NAC appear to be a result of its ability to support glutathione production and the reduction of disulfide bridges between glycopolypeptides in mucus, respectively (12, 28). More recently, in a study by Witschi et al. (1995), NAC was used in the treatment of AIDS because of its ability to inhibit activation of NF-κB, which is a required step in HIV replication cycle (78, 55). In light of this information, there exists the exciting possibility that NAC may prove efficacious in the treatment of hRV colds.

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